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Challenges and opportunities in orchid ecology and conservation

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Challenges and opportunities in orchid ecology and conservation

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Table of contents

05 Editorial: Challenges and opportunities in orchid ecology and conservation
Pavel Kindlmann, Tiiu Kull and Dennis Whigham

08 Range Size and Niche Breadth as Predictors of Climate-Induced Habitat Change in *Epipactis* (Orchidaceae)
Alexandra Evans and Hans Jacquemyn

19 Orchid diversity along an altitudinal gradient in the central Balkans
Vladan Djordjević, Spyros Tsiftsis, Pavel Kindlmann and Vladimir Stevanović

34 Dominant *Dendrobium officinale* mycorrhizal partners vary among habitats and strongly induce seed germination *in vitro*
Liyue Zhang, Kento Rammitsu, Kenshi Tetsuka, Tomohisa Yukawa and Yuki Ogura-Tsujita

46 Delimiting species in the taxonomically challenging orchid section *Pseudophrys*: Bayesian analyses of genetic and phenotypic data
Nina Joffard, Bruno Buatois, Véronique Arnal, Errol Véla, Claudine Montgelard and Bertrand Schatz

59 Shade and drought increase fungal contribution to partially mycoheterotrophic terrestrial orchids *Goodyera pubescens* and *Tipularia discolor*
Melissa K. McCormick, Kerry L. Good, Thomas J. Mozdzer and Dennis F. Whigham

70 Host tree species effects on long-term persistence of epiphytic orchid populations
Adriana Ramírez-Martínez, Tamara Ticktin and Demetria Mondragon

82 Will Greenland be the last refuge for the continental European small-white orchid?Niche modeling of future distribution of *Pseudorchis albida*
Marta Kolanowska, Sławomir Nowak and Agnieszka Rewicz

96 Taxonomic revision of *Sobralia* section *Racemosae* Brieger (*Sobralieae*, Orchidaceae)
Przemyslaw Baranow, Dariusz Szlachetko and Pavel Kindlmann

123 The effect of habitat transformation on a twig epiphytic orchid: Evidence from population dynamics
Nhora Helena Ospina-Calderón, Raymond L. Tremblay, Alba Marina Torres and Nicola S. Flanagan

135 Floral and genetic divergence across environmental gradients is moderated by inter-population gene flow in *Platanthera dilatata* (Orchidaceae)
Lisa E. Wallace and Marlin L. Bowles

151 Diversity and specificity of orchid mycorrhizal fungi in a leafless epiphytic orchid, *Dendrophylax lindenii* and the potential role of fungi in shaping its fine-scale distribution
Lynnaun J. A. N. Johnson, Michael E. Kane, Lawrence W. Zettler and Gregory M. Mueller

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Editorial: Challenges and opportunities in orchid ecology and conservation

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Editorial on the Research Topic
Challenges and opportunities in orchid ecology and conservation

Understanding diversity patterns and how they are affected by global change are topics of active discussion in biodiversity research. In response to species declines, it is important to not only understand patterns of diversity but also develop a knowledge base for use in species conservation. We still do not know, for example, the abiotic and biotic requirements for population persistence for most species.

Orchid ecology and conservation are the subjects of this Research Topic. We focus on orchids because the family has the most species and more than 50% of the species that have been assessed fall into one or more risk categories. Given the large number of orchid species, relatively few have been studied in detail. As a result, it is difficult to determine the best approach for conserving species. Given the increasing threats to orchids globally, the editors chose to focus on orchid ecology and conservation and the contributing authors have provided a range of relevant topics.

Orchid-fungal interactions are the focus of three papers

Most orchids are mixotrophic, indicating that they obtain resources from fungal interactions as well as photosynthesis. Orchid responses to changes in environmental conditions have rarely been investigated, especially in terms of orchid-fungal interactions. McCormick et al. experimentally manipulated light and soil moisture for two terrestrial species and used isotopes to compare changes in carbon and nitrogen. They found that reductions in light and soil moisture increased the dependence of both species on fungal carbon and nitrogen.

Zhang et al. identified orchid mycorrhizal fungi (OMF) associated with *Dendrobium officinale*, an orchid of medicinal value. Almost 84% of the OMF identified from plants at six sites were in the Tulasnellaceae and Serendipitaceae families and the relative abundance of the two fungi varied between plants that grew on rocks versus plants on trees. They demonstrated that two of the fungi supported the germination and growth of *Dendrobium*,

providing evidence that there are differences among OMF in their ability to support germination and growth. They suggested that future research should focus on the use of *in situ* seed baiting as a method for obtaining OMF from protocorms that are most likely to support the early growth stages of orchids in nature.

Like terrestrial species, epiphytic orchids interact with mycorrhiza. Johnson et al. identified the mycorrhiza associated with the Ghost Orchid (*Dendrophylax lindenii*) and other epiphytic orchids. They also compared the fungi on the bark of trees that had the Ghost Orchid with bark from trees where the orchid did not occur. They found that the fungus associated with *Dendrophylax* was very specific and was a species of *Ceratobasidium* that was not found in other epiphytes. Furthermore, they found that plants grown in the lab had a lower abundance of *Ceratobasidium* than plants that occurred naturally. Their results provide evidence that the distribution of fungi influences the distribution of the Ghost Orchid.

Surprisingly, taxonomy had the second-highest number of contributions

Likely the result of the rapid development of powerful computers and sophisticated genetic and molecular biology methods, taxonomy is becoming a Cinderella in systematic research, including orchids. An increased knowledge of orchid identity is, however, necessary to support ecological and conservation research.

Baranow et al. revised the *Sobralia*, section *Racemosae*, a large and diverse genus that can be divided into four sections and some informal species groups based mainly on inflorescence architecture. The section *Racemosae* has species with an elongated inflorescence with distinct internodes, but the species are often similar and easily misidentified, especially with herbarium specimens. Baranow et al. present species' morphological characteristics, keys for identification, ecological data, and distribution maps. They describe a new species, *Sobralia gambitana*, and a neotype for *S. hoppii* Schltr. is proposed.

Tools that can integrate genetic and phenotypic data in taxonomic studies have been recently developed and were used by Joffard et al. to investigate species in the genus *Pseudophrys*. Using an approach termed iBPP they identified four groups of species rather than 12 and they merged two groups of species. They demonstrated that phenotypic data are particularly informative in section *Pseudophrys*, and the approach that they used improves species identification. They recommended that an integrative taxonomic approach holds great promise for conducting taxonomic revisions in other orchid groups.

Climate change, a globally important topic, was the focus of two papers

Evans and Jacquemyn examined the impact of climate change on 14 *Epipactis* species with a focus on species that are habitat specialists or generalists. Species with a wide distribution are more capable of shifting habitats but only if they can fully expand into habitats at the leading edge of their distributions. This study provides valuable insights into how terrestrial orchid species with differing niche breadths may respond to climate change.

Kolanowska et al. investigated the impact of climate change on the future distribution of the small-white orchid (*Pseudorchis albida*). The niche model that they used predicted that although the number of suitable niches will increase significantly in Greenland, suitable habitats will severely decline in continental Europe. Importantly, their research indicated that global warming might have an opposite effect on the pollinators of *P. albida* because of insect habitat loss, but some pollinators are expected to remain within the orchid's potential geographical range, supporting its long-term survival.

The remaining four papers are examples of topics that are relevant to a more complete understanding of orchid ecology and conservation

"Can orchids occur in landscapes that have been modified by human activities"? That question is the topic addressed by Ospina-Calderón et al. They studied the distribution of epiphytes in undisturbed forests in the Andes and their distribution on shade trees in coffee plantations and trees in a grassland matrix. They collected data over 2 years and constructed demographic transition matrices with transition probabilities calculated using the Bayesian approach. Population growth rates were higher on trees in coffee plantations compared with forests. Although the orchids also occurred on trees in the grassland matrix, the authors suggested that those populations represented a temporal phase that would not be sustainable.

Wallace and Bowles explored the topic of genetic variation as a function of gene flow in *Spiranthes dilitata*, a widespread species in Alaska. They found evidence for small-scale genetic variation associated with different habitats and differences in the ability of pollinators to pollinate different morphotypes. This research provided clear evidence that evolution in orchids can occur at spatially small scales and can be influenced by pollinators.

Ramírez-Martínez et al., like Wallace and Bowles, found that differences in species performance can operate at small scales in response to habitat conditions. They compared the population dynamics of two epiphytic species in Mexico that occurred on deciduous and semi-deciduous trees. It was demonstrated that in years with normal rainfall, there were no differences in plant performance, but during dry years, *Alamania punicea* was more vulnerable to drying conditions—most likely because it has smaller pseudobulbs that have less storage capacity. This research provides evidence that climate change will potentially influence the population dynamics of epiphytic orchids.

Djordjevic et al. sampled orchids along an elevation gradient in the Balkans, with a focus on the belowground features of the different species and their pollination. Results showed that species

diversity peaked at 900–1,000 m, with variations in distribution patterns for different life history traits and habitat types. Deceptive orchids were most abundant at lower and mid-elevations. By contrast, rewarding orchids were more common at mid to high elevations. This study demonstrates that data that link orchid species to habitats are important for conservation efforts.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Range Size and Niche Breadth as Predictors of Climate-Induced Habitat Change in *Epipactis* (Orchidaceae)

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While there is mounting evidence that ongoing changes in the climate system are shifting species ranges poleward and to higher altitudes, responses to climate change vary considerably between species. In general, it can be expected that species responses to climate change largely depend on how broad their ecological niches are, but evidence is still scant. In this study, we investigated the effects of predicted future climate change on the availability of suitable habitat for 14 *Epipactis* (Orchidaceae) species, and tested whether habitat specialists would experience greater changes in the extent of their habitats than habitat generalists. We used Maxent to model the ecological niche of each species in terms of climate, soil, elevation and land-use and projected it onto climate scenarios predicted for 2061–2080. To test the hypothesis that temperate terrestrial orchid species with small ranges or small niche breadths may be at greater risk under climate change than species with wide ranges or large niche breadths, we related niche breadth in both geographic and environmental space to changes in size and location of suitable habitat. The habitat distributions of half of the species shifted northwards in future projections. The area of suitable habitat increased for eight species but decreased for the remaining six species. If expansion at the leading edge of the distribution was not possible, the area of suitable habitat decreased for 12 species. Species with wide niche breadth in geographic space experienced greater northwards expansions and higher habitat suitability scores than species with small niche breadth. Niche breadth in environmental space was not significantly related to change in habitat distribution. Overall, these results indicate that terrestrial orchid species with a wide distribution will be more capable of shifting their distributions under climate change than species with a limited distribution, but only if they are fully able to expand into habitats at the leading edge of their distributions.

Keywords: climate change, ecological niche, ENMTools, *Epipactis*, Maxent, range size

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INTRODUCTION

Climate plays an important role in the distribution of plant and animal species and in light of the global climate crisis, the effects of changing climate on plant species distributions is a prominent topic in ecology (Chen et al., 2011; Tayleur et al., 2015; Lehikoinen and Virkkala, 2016). In order to survive climate change, species must either shift their range limits to environments that are able to support them or adapt to the new conditions in their current environments (Thuiller, 2007; Kelly and Goulden, 2008; Scheffers et al., 2016; Ash et al., 2017). Predicting how a species' suitable habitat alters due to climate change is necessary when planning its long-term conservation, but can be difficult because of the wide variety of habitat needs and tolerances among species.

Species differ in their responses to climate change based on how broad their ecological niches are (Thuiller et al., 2005). Previous research has already shown that species within a genus can vary considerably in habitat preferences and distributions (Brown et al., 1996; Grossenbacher and Whittall, 2011; Anacker and Strauss, 2014; Duffy and Jacquemyn, 2019). Habitat generalists tend to have wider ranges of conditions where they can survive, grow and reproduce and are therefore assumed to be more adaptable to environmental change (Marvier et al., 2004; Thuiller et al., 2005). Specialist species, on the other hand, tend to have more specific environmental requirements and therefore can only occupy a narrow ecological niche. It is expected that species which have narrow temperature or precipitation tolerances are the most likely to be affected by climate change (Slatyer et al., 2013). However, empirical evidence is still limited (Shay et al., 2021) and for many species we do not know the factors that limit their distributions, whether leading edge expansions are sustainable, or how these species respond to climate change. Gaining a better understanding of the physical factors underlying the distribution of organisms is crucial to predict how species will respond to climate change (Hagsater et al., 1996; Tsiftsis et al., 2008).

Although orchids are generally considered rare and have small population sizes (Tremblay et al., 2005; Otero and Flanagan, 2006; Shefferson et al., 2020), there is often large variation in range size and environmental tolerance between species, both within and among orchid genera (McCormick and Jacquemyn, 2014; Evans and Jacquemyn, 2020). What drives variation in orchid species range size is not well known, but is likely a combination of factors including niche breadth, species age, niche availability and range position (Sheth et al., 2020). Previous research has shown that orchid species vary in their dependence on specific abiotic environmental conditions, with some species being limited primarily by temperature and precipitation (McCormick et al., 2009; Djordjević et al., 2016; Evans et al., 2020) and others being limited more by local growth conditions related to bedrock and soil (Bowles et al., 2005; Tsiftsis et al., 2008; Bunch et al., 2013). Consequently, specialist orchid species are often associated with the habitat types that arise from the specific combinations of these abiotic characteristics, from coastal dunes to temperate forests, and the spatial extent of these habitats therefore can limit the range of the species they support (McCormick and Jacquemyn, 2014; Djordjević and Tsiftsis, 2022). Species traits related to growth and reproduction in a habitat, such as root system and pollination, can affect spatial distribution. For example, wide spatial distributions of orchids in the Czech Republic were associated with a rhizomatous root system (Štípková et al., 2021), and the wide variety of pollinators utilised by the terrestrial orchid *Epipactis helleborine* is likely an important contributor to its large range and ability to colonise various habitats (Rewicz et al., 2017).

Recently, it has become clear that weather conditions can have a strong impact on orchid population dynamics, suggesting that changing climatic conditions have the potential to affect the geographic distribution of orchids. For example, climatic changes during the last three decades have been shown to have a positive effect on the survival of the terrestrial orchid *Himantoglossum hircinum* at the northern edge of its population in the United Kingdom (van der Meer et al., 2016) and warmer winter weather conditions have also been shown to be beneficial to German populations of this species (Pfeifer et al., 2006). Williams et al. (2015) demonstrated that the population dynamics, vital rates and reproduction of the lady orchid (*Orchis purpurea*) at the northern edge of its distribution were affected by seasonal temperature and precipitation and, specifically, that milder winters and wetter springs were beneficial for its population growth. These results suggest that a warmer climate will generally benefit orchids at the northern edges of their distributions. A recent modelling study has indeed shown that predicted changes in climatic conditions increased habitat suitability available to three *Orchis* species by 2050 at the northern edge of their distribution (Evans et al., 2020). However, given that these species showed very similar distribution areas and often co-occur, such a generalisation may not be appropriate and it remains unclear how differences in range size or environmental niche breadth predict vulnerability under global change (Shay et al., 2021).

In this study, we tested the hypothesis that orchid species with small ranges or small niche breadths may be at greater risk under climate change than species with wide ranges or large niche breadths. We used the orchid genus *Epipactis* as a study system. *Epipactis* is a widespread genus occurring throughout the European and Asian continents with 37 species according to the The Euro+Med Plantbase Project (2022) although the results of phylogenetic research in recent years has brought into question the status of many species (Sramkó et al., 2019; Bateman, 2020). Previous research has shown that among fourteen European *Epipactis* species, range size differed by more than three orders of magnitude between species with the smallest and largest ranges (Evans and Jacquemyn, 2020). The distribution of small-range species was strongly associated with local habitat conditions and landscape structure, while that of large-range species was more associated with climatic conditions (Evans and Jacquemyn, 2020). However, whether the habitat distributions of generalist species are more strongly affected by climate change than small-range, specialist species, is yet unknown. Specifically, we investigated how the habitat of the same fourteen *Epipactis* species would be affected by changes in temperature and precipitation in Europe predicted for 2061–2080, and assessed whether species with

small ranges or narrow ecological niches would suffer greater changes in size and latitudinal position of habitat than species with large ranges.

MATERIALS AND METHODS

Study Species and Occurrence Data

The genus *Epipactis* contains a large number of terrestrial orchids which vary greatly in distribution area and habitat type (Sramkó et al., 2019; Evans and Jacquemyn, 2020). Some species (e.g., *E. dunensis* and *E. albensis*) have very localised distributions and are restricted to particular habitats such as coastal dunes and beech forests, whereas others (e.g., *E. helleborine* and *E. atrorubens*) are widespread and can tolerate a relatively wide range of habitat conditions. There are several ecotypes of *E. helleborine* that can be found in specific habitats such as coastal dunes and forests (Jacquemyn et al., 2018). Species are autogamous, allogamous, or facultative allogamous (Claessens and Kleynen, 2011; Brys and Jacquemyn, 2016). The numerous seeds produced by *Epipactis* species are very small, dispersed by wind, and rely on the presence of mycorrhizal fungi in the soil to germinate and establish (Bidartondo and Read, 2008; Smith and Read, 2010; McCormick and Jacquemyn, 2014; Jacquemyn et al., 2018; Xing et al., 2020). Differences in mycorrhizal communities between localities may contribute to reproductive isolation and spatial distribution of *Epipactis* species and populations (Ogura-Tsujita and Yukawa, 2008; Jacquemyn et al., 2016, 2018; but see Těšitelová et al., 2012).

Records of each species' occurrence from 2000 to 2020 on the continent of Europe were obtained from the online database GBIF[1] (**Supplementary Material**). We discarded records with missing GIS coordinates, ambiguous species identification or with coordinates with a spatial resolution lower than 100 m. This resulted in between 31 (*Epipactis lusitanica*) and 45,354 (*E. helleborine*) occurrences per species. Records for each species were aggregated into 10 km^2 grid cells to reduce the effects of spatial clustering resulting from sampling bias, by extracting the centre coordinates of each grid cell in which the species was recorded (**Supplementary Table 1**). Processing of occurrence data was performed in QGIS v3.4.9 (QGIS Development Team, 2019).

Ecogeographic Variables

Previous studies have shown that land cover, bedrock, precipitation, and temperature are important variables predicting the distributions of some *Epipactis* species (Tsiftsis et al., 2008; Djordjević et al., 2016; Evans and Jacquemyn, 2020). We therefore used nine raster-format predictor variables with <0.5 correlation with one other. Two of the 19 bioclimatic variables available at the WorldClim v2 online database (Fick and Hijmans, 2017[2]) were used in our model, mean annual temperature and annual precipitation, projected for the near-present climate (1970–2000). These two variables were chosen because they are the most representative of the mean climate of an area, and are therefore appropriate for a continent-wide distribution study such as this. We also obtained the mean annual temperature and annual precipitation rasters predicted for the years 2061–2080 predicted by two Shared Socio-economic Pathways (SSPs), SSP 2-4.5 and SSP 5-8.5 from WorldClim.[3] SSP 2-4.5 models the climate in a scenario where greenhouse gas emissions are at their highest (∼44 GT CO_2) in 2040 and then decrease to 9.6 GT in 2100, while in SSP 5-8.5, emissions increase steeply until the year 2080 (∼130 GT) before starting to stabilise and decrease (Riahi et al., 2017). Maps of the distribution of temperature and precipitation values in Europe were created by calculating the mean temperature for each cell of a 50 km^2 cell grid of Europe and summarising the values in QGIS.

The other seven variables used were the same as those used to model *Epipactis* species in Evans and Jacquemyn (2020). These include the first two components of two PCAs run on two topsoil datasets (physical and biochemical measures) acquired through the European Soil Data Centre (ESDAC) (Hiederer, 2013; Ballabio et al., 2019), dominant bedrock from the ESDAC database (Van Liedekerke et al., 2006), Corine Land Cover (CLC) from the Copernicus programme of the European Environmental Program (Heymann, 1994) and elevation (Amatulli et al., 2018). All raster processing was performed in RStudio v4.0.2 (R Core Team, 2021).

Ecological Niche Modelling

Defining and quantitatively comparing plant niches can be achieved using ecological niche models (ENMs). Ecological niche modelling has been applied successfully to numerous species to investigate ecological niches and to assess the impacts of climate change and land use on species ranges (Guisan and Thuiller, 2005). We used the programme Maxent v3.4.1. (Phillips et al., 2017) to model the effects of predicted climate change on species' habitats. Maxent is a popular ENM tool that uses species occurrence data and environmental rasters to calculate a Gibbs value for each pixel of the study area, or the probability that the pixel has suitable habitat conditions for the species (Phillips, 2005) and performs well in comparison to other modelling methods (Elith et al., 2006; Phillips and Dudík, 2008; Valavi et al., 2021). Maxent creates habitat suitability maps over the study area from these data, as well as a table of the contribution of each predictor variable to the distribution of suitable habitat for each species. The choice of Maxent settings was informed by Barbet-Massin et al. (2012) and Merow et al. (2013). Each model was run using a random seed and 100 bootstrap replicates with 75% of the data used to train the model and 25% to test it. The rest of the settings were left as the default (convergence threshold of 0.00001, regularisation threshold of 1 and a maximum of 10,000 background points) and allowed for linear, quadratic, product and hinge features to be chosen automatically, producing a cloglog output. The models were run for the current climatic features and projected onto the SSP climate data to produce

[1] www.GBIF.org

[2] https://www.worldclim.org/data/worldclim21.html

[3] https://www.worldclim.org/data/cmip6/cmip6climate.html

separate environmental niche map outputs for the current and future climate conditions.

Data Analysis

For each habitat suitability map, the mean Gibbs value with standard error was calculated for every latitudinal interval of 0.5 decimal degree of the study area (Europe) using the Zonal Statistics tool in QGIS. We ran a Wilcoxon signed rank test on these mean Gibbs values multiplied by their corresponding latitudes to test whether the suitable habitat of each species will shift in latitude in future climate scenarios. Before running the Wilcoxon tests, the set of Gibbs values for each climate scenario was centred by dividing by the mean to eliminate the influence of different mean Gibbs values between climate scenarios and test only for shifts in latitude.

The continuous probabilistic maps produced by Maxent were converted into binary presence maps using the Maximum Test Sensitivity plus Specificity (MTSS) value of each species as a threshold. The pixels with a Gibbs value of greater than the MTSS were extracted and plotted as a new map for each species, with each pixel representing the species being present at that location. The total numbers of pixels occupied by these habitat distribution maps were compared between current and future climate scenarios. The overlapping pixels between the current and future distributions (i.e., pixels for which occurrence equalled one for both maps) of each species were extracted and counted to provide a measurement of the area suitable for a species if it were unable to expand into any newly available areas created by future climate change.

Mean species occurrence per climate scenario and per species, for both the continuous Gibbs values and the number of pixels occupied of the binary maps, were compared between the climate scenarios using Kruskal–Wallis and Dunn tests with a Holm correction or ANOVA and Tukey tests if the data were normally distributed.

We calculated Levins' B2 values of niche breadth in geographic ($B2_{geo}$) and environmental space ($B2_{env}$) for each species using the functions raster.breadth and env.breadth, respectively, in the ENMTools 1.0.5 R package (Warren et al., 2021). B2 ranges from 0 to 1, with values closer to 0 representing narrow (specialised) niche breadth and values closer to 1 representing wide (generalised) niche breadth. Finally, we investigated whether species niche breadth predicts changes in distribution in response to climate change by dividing the range change of a species from current to future scenarios, converting to the proportional change for each species, and comparing these values to each species' B2 value using ordinary least-squares regression. Ordinary least-squares regression was also used to compare the Levins' B2 values between geographic and environmental space. The difference in niche breadth between species with positive range changes and negative range changes in response to habitat change was investigated using Kruskal–Wallis tests. The effect of mating system on response to climate change was tested by comparing the mean changes in latitudinal habitat distribution and proportional range size between autogamous, allogamous and facultative autogamous species, using Kruskal–Wallis tests. All analyses were performed in RStudio v4.0.2 (R Core Team, 2021).

RESULTS

The mean temperature in continental Europe will increase from a near-current mean of 9.21 ± 0.10°C (standard error) to 12.16 ± 0.09°C for SSP 2-4.5 and to 13.42 ± 0.09°C for SSP 5-8.5 (**Figure 1**) predicted for the years 2061–2080. The mean annual precipitation will increase slightly in the future projections, with a current mean of 742.74 ± 5.86–761.95 ± 5.86 mm^3 for SSP 2-4.5 and 761.43 ± 5.93 mm^3 for SSP 5-8.5.

The mean current habitat suitability or probability of occurrence (Gibbs *p*-value) predicted by the Maxent model ranged from 0.0026 ± 0.0006 for *E. lusitanica* to 0.29 ± 0.022 for *E. helleborine*. When the model was projected for the climate of 2061–2080, there was no significant difference in mean habitat suitability between the current climatic conditions and either of the two future climate scenarios ($\chi^2 = 0.18$, p-value = 0.91). Although the mean species' habitat suitability did not change, when species were tested individually, the habitat suitability of *E. helleborine*, *lusitanica*, *phyllanthes*, and *tremolsii* significantly increased under both SSP scenarios, and *E. albensis* increased significantly for SSP 5-8.5 (**Table 1**; see **Supplementary Table 2** for mean Gibbs values). Seven species (*E. albensis*, *fageticola*, *kleinii*, *leptochila*, *microphylla*, *muelleri*, and *tremolsii*) demonstrated significant northwards shifts in their habitat distributions in both future climate scenarios (**Table 2** and **Supplementary Material** for illustration of individual range shifts).

The area of suitable habitat available (pixels where the Gibbs *p*-value was above the species' MTSS threshold) increased for eight species (*E. albensis*, *dunensis*, *fageticola helleborine*, *lusitanica*, *microphylla*, *phyllanthes*, and *tremolsii*) in the future scenarios, but decreased for the remaining six species (*E. atrorubens*, *kleinii*, *leptochila*, *muelleri*, *palustris*, and *purpurata*; **Table 3**). For species that responded positively to the climatic changes, the increase in habitat ranged between 5 and 1000% (*E. dunensis* and *E. lusitanica*), while for those that responded negatively, decrease in habitat area ranged between 5% (*E. kleinii*, *muelleri*, and *palustris*) and 88% (*E. purpurata*).

Overlap in habitat distribution areas between current and future climate scenarios was fairly high, ranging from 57 to 100% for SSP 2-4.5 and 33 to 100% for SSP 5-8.5, except for *E. purpurata* which showed notably low overlap (16 and 4% for the two scenarios, respectively; see **Supplementary Data** for range values). The change in habitat area experienced by species if they would not be able to track the climate in the future decreased by up to 95% (*E. purpurata*) for all except two species, *E. lusitanica* and *E. phyllanthes*, which showed no decrease in distribution area (100% overlap, only expansion).

The niche breadth values of Levins' $B2_{geo}$ (in geographic space) ranged from 0.39 for *E. fageticola* to 0.85 for *E. palustris*, while $B2_{env}$ ranged from 0.16 for *E. dunensis* to 0.90 for *E. lusitanica* (see **Supplementary Material**). B2 values in geographic and

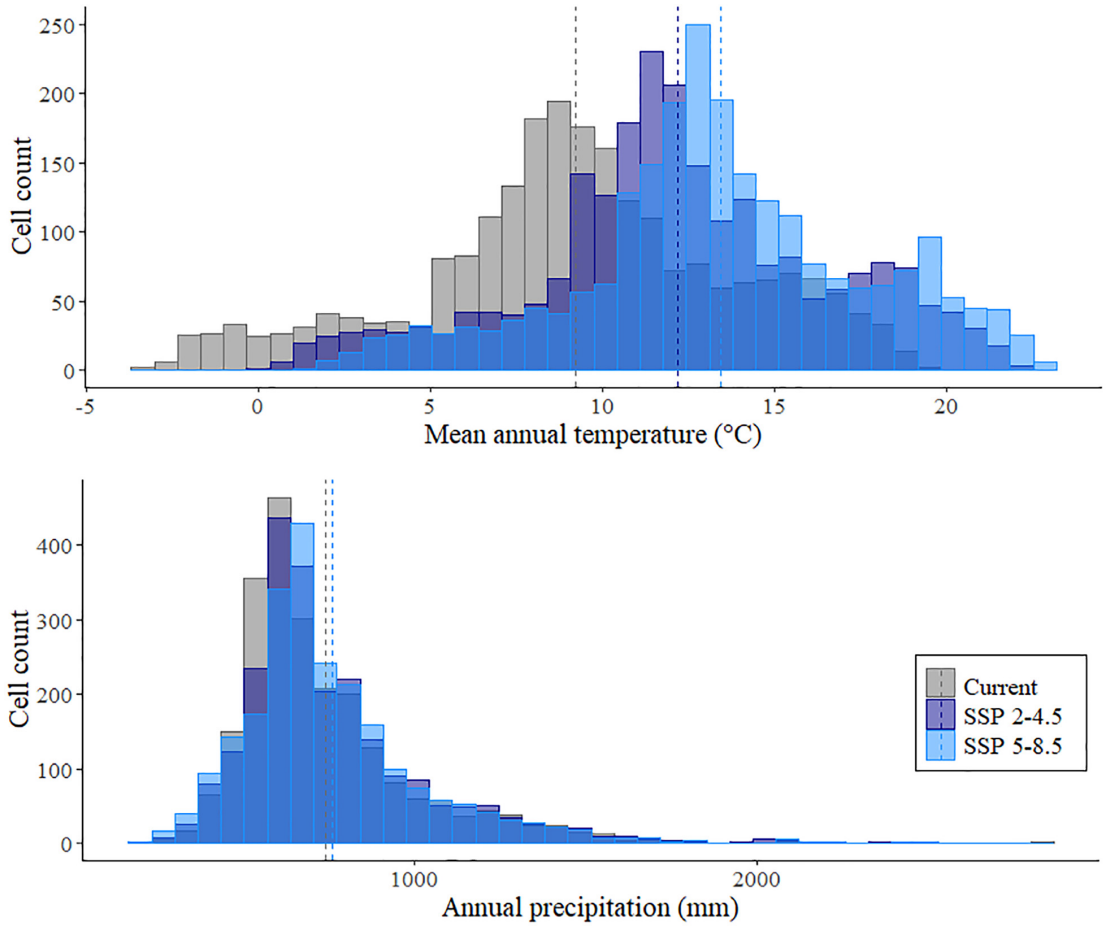

FIGURE 1 | Distribution of mean annual temperature and annual precipitation in Europe predicted for the current climate, and projected to occur in the years 2061–2080 under two Shared Socio-economic Pathways (SSP2-4.5 and SSP5-8.5). Bars represent the number of 50 km grid squares of Europe with a corresponding mean temperature or precipitation value, and dotted lines represent the mean values.

TABLE 1 | Change in mean Gibbs values (habitat suitability) for *Epipactis* species from current to future (2061–2080) climate scenarios (SSP 2-4.5 and SSP 5-8.5) and results of Dunn tests comparing current and future mean Gibbs *p*-values (showing only results for significant differences, in bold, and marginally significant differences, in italics).

Species	Change mean Gibbs *p*		Current – SSP 2-4.5		Current – SSP 5-8.5	
	SSP 2-4.5	SSP 5-8.5	*Z*	*p*	*Z*	*p*
E. albensis	0.0025	0.0037	−2.1573	*0.0620*	−3.1508	**0.0049**
E. atrorubens	−0.0196	−0.0378				
E. dunensis	0.0007	0.0059	−0.3820	0.7024	−2.3288	*0.0596*
E. fageticola	0.0003	0.0002				
E. helleborine	0.0645	0.0791	−2.2945	**0.0435**	−2.5807	**0.0296**
E. kleinii	−0.0002	−0.0008				
E. leptochila	−0.0038	−0.0060				
E. lusitanica	0.0064	0.0102	−3.4435	**0.0011**	−4.9054	**<0.0001**
E. microphylla	0.0024	0.0008				
E. muelleri	−0.0014	−0.0065				
E. palustris	0.0193	0.0136				
E. phyllanthes	0.0056	0.0084	−2.7392	**0.0123**	−3.8179	**0.0004**
E. purpurata	−0.0113	−0.0182				
E. tremolsii	0.0044	0.0065	−2.5263	**0.0231**	−3.7593	**0.0005**

TABLE 2 | Latitudinal shifts in habitat distribution from current to future climate scenarios and results of Wilcoxon tests of differences in habitat distributions (showing only results for significant differences).

Species	Change in mean Gibbs *p**latitude		Current – SSP 2-4.5		Current – SSP 5-8.5	
	SSP 2-4.5	SSP 5-8.5	*W*	*p*	*W*	*p*
E. albensis	86792.3	179258.2	1016	0.0091	918	0.0019
E. atrorubens	370209.9	558240.5				
E. dunensis	7321.7	3531.9				
E. fageticola	45463.3	112859.5	986	0.0058	985	0.0057
E. helleborine	319826.6	459016.5				
E. kleinii	49895.4	165549.1	998	0.0069	904	0.0015
E. leptochila	155493.8	204799.6	1121	0.0369	1087	0.0241
E. lusitanica	105798.9	199849.8				
E. microphylla	243820.9	385056.3	1076	0.0208	1110	0.0322
E. muelleri	246742.1	397285.9	1063	0.0175	1081	0.0222
E. palustris	332171.1	506155.0				
E. phyllanthes	91731.5	164969.6				
E. purpurata	433625.6	748751.2				
E. tremolsii	253596.0	412612.4	802	0.0002	829	0.0004

environmental spaces were not correlated with one another (p-value = 0.74). The means Gibbs value of habitat suitability was positively correlated with $B2_{geo}$ for both current ($R^2 = 0.33$, $F_{1,12} = 7.52$, p-value = 0.012) and future (SSP 2-4.5: $R^2 = 0.50$, $F_{1,12} = 13.81$, p-value = 0.0029; SSP 5-8.5: $R^2 = 0.53$, $F_{1,12} = 15.81$, p-value = 0.0020) climate projections (**Figure 2A**). Similarly, $B2_{geo}$ had a positive relationship with range size for current ($R^2 = 0.32$, $F_{1,12} = 7.11$, p-value = 0.021) and future (SSP 2-4.5: $R^2 = 0.53$, $F_{1,12} = 15.41$, p-value = 0.0020; SSP 5-8.5: $R^2 = 0.52$, $F_{1,12} = 13.19$, p-value = 0.0034) climate projections (**Figure 2B**). Species with higher $B2_{geo}$ values also experienced greater changes in Gibbs values between current and future climate scenarios (**Figure 2C**; SSP 2-4.5: $R^2 = 0.68$, $F_{1,12} = 17.93$, p-value = 0.0039; SSP 5-8.5: $R^2 = 0.43$, $F_{1,12} = 7.09$, p-value = 0.032). There was a positive relationship between $B2_{geo}$ and the change in mean Gibbs value between current climate and SSP 2-4.5 multiplied by latitude (**Figure 2D**; $R^2 = 0.24$, $F_{1,12} = 4.99$, p-value = 0.045), indicating that species with higher $B2_{geo}$ values would experience a greater northwards shift in suitable habitat than those with low $B2_{geo}$ values, if they were able to track the suitable climate. This was also marginally significant for SSP 5-8.5 ($R^2 = 0.18$, $F_{1,12} = 3.77$, p-value = 0.076). No comparisons involving $B2_{env}$ were significant at $\alpha = 0.05$, but marginally significant positive relationships were detected between $B2_{env}$ and proportional range change (proportional to the species' current range) from current to future climate scenarios (SSP 2-4.5: $R^2 = 0.31$, $F_{1,12} = 4.20$, p-value = 0.086; SSP 5-8.5: $R^2 = 0.17$, $F_{1,12} = 3.64$, p-value = 0.081). Species with higher $B2_{env}$ also showed some evidence for experiencing a greater northwards shift in suitable habitat from current to SSP 5-8.5 climate ($R^2 = 0.16$, $F_{1,12} = 3.77$, p-value = 0.076). The mean proportional range change (with and without tracking) and change in latitudinal habitat distribution in response to climate change in either SSP scenario were not significantly different between mating systems (p-value > 0.05).

DISCUSSION

In this study we investigated how the distribution of suitable habitat of *Epipactis* species would be affected by predicted climate change and whether species with small ranges or narrow niche breadths are at greater risk from climate change than species with wide ranges or large niche breadths. Our results showed that the habitat available increased on the leading (northern) edge of the distribution for half of the species but decreased for the

TABLE 3 | Changes in area of suitable habitat above the Maximum Test Sensitivity and Specificity threshold of each species from the current climate conditions to future climate scenarios (SSP 2-4.5 and SSP 5-8.5), as well as changes if species are unable to expand their ranges into the climatic envelope of future scenarios (climate tracking).

Species	Proportional range change		Proportional range change without tracking	
	SSP 2-4.5	SSP 5-8.5	SSP 2-4.5	SSP 5-8.5
E. albensis	1.9094	3.4189	−0.0025	−0.0214
E. atrorubens	−0.3250	−0.5481	−0.4259	−0.6711
E. dunensis	0.0554	0.5265	−0.0019	−0.0136
E. fageticola	0.2203	0.5554	−0.0059	−0.2330
E. helleborine	0.2001	0.1391	−0.1960	−0.2970
E. kleinii	−0.0476	−0.3597	−0.1228	−0.4138
E. leptochila	−0.3257	−0.4843	−0.3862	−0.5520
E. lusitanica	5.9117	10.8262	< 0.0001	< 0.0001
E. microphylla	0.3052	0.2582	−0.2469	−0.4614
E. muelleri	−0.0467	−0.2476	−0.3005	−0.5037
E. palustris	−0.0479	−0.1842	−0.2636	−0.4495
E. phyllanthes	1.5237	2.3245	< 0.0001	< 0.0001
E. purpurata	−0.6798	−0.8866	−0.8357	−0.9555
E. tremolsii	0.6798	1.0058	−0.1054	−0.1888

Range changes are reported as proportional to the current range.

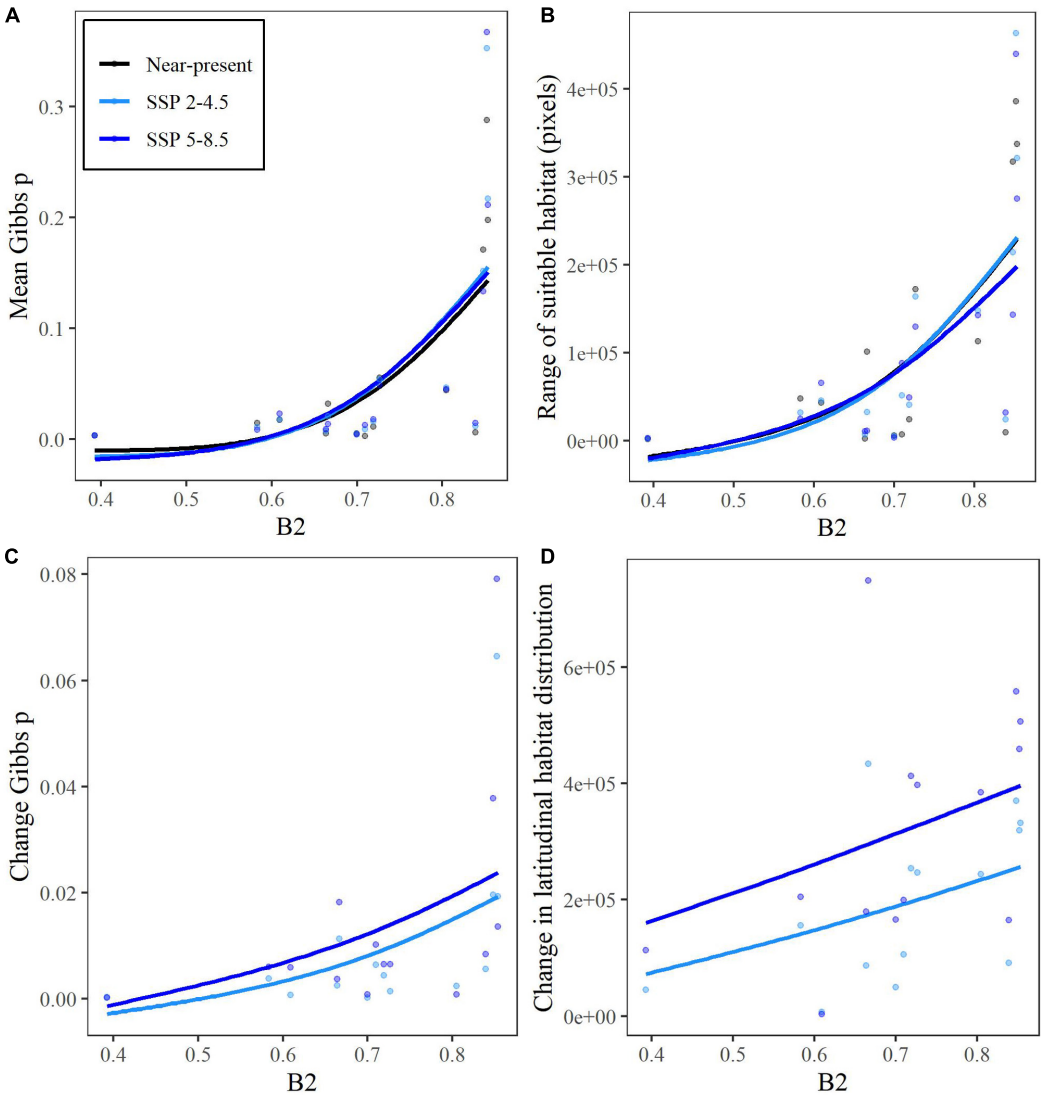

FIGURE 2 | Relationships between Levins' B2 measure for niche breadth in geographic space and **(A)** the mean Gibbs *p*-value (habitat suitability), **(B)** the spatial range of suitable habitat, **(C)** change in mean Gibbs *p* from current to future climate scenarios, and **(D)** change in latitudinal habitat distribution of 14 *Epipactis* species. Two climate scenarios were used, SSP 2-4.5 and SSP 5-8.5 to predict the climate for 2061–2080.

remaining species, and decreased for all but two species if climate tracking was not possible. Levins' B2 metric for niche breadth in geographic space was highly correlated with the spatial extent and mean Gibbs value (habitat suitability) of species habitat distributions and species with a higher B2 value were predicted to experience a greater northwards expansion in response to climate change. We did not detect significant effects of Levins' B2 in environmental space, although there was marginally significant patterns similar to those of B2 in geographic space.

Impact of Climate Change on the Distribution of *Epipactis* in Europe

Although there was no change in the mean Gibbs value of the 14 species between current and future climate scenarios, the Gibbs values for the majority of the species individually was predicted to increase in 2061–2080. The area of suitable habitat increased into the north for some species and decreased in the south for most species in the future, resulting in a mean northern shift in habitat. When expansion into the north (climate tracking) was restricted, the area of habitat decreased by up to 95% for all except two small-range species.

Despite the expectation that species with narrow environmental tolerances are most threatened by climate change, in the case of *Epipactis*, the habitats of most of the small-range localised species that we investigated were predicted to increase with future climate change. Some northern hemisphere herbaceous species benefit from increased temperatures at the northern edge of their distribution through increased population growth, which in turn can lead to an increase in geographic

range at this edge (Bremer and Jongejans, 2009). This includes orchids such as *H. hircinum* where climatic changes in the United Kingdom were shown to be partially responsible for the species' expansion between 1991 and 2001, as well as for projected future scenarios (van der Meer et al., 2016). Similarly, Ongaro et al. (2018) predicted that the habitat range for nine orchid species will increase by 2070 on the island of Sardinia, although the probability of presence in the newly colonised habitats was not predicted to increase. However, Vogt-Schilb et al. (2015) found that the distributions of many orchids in Western Europe have declined in the last two decades due to land-use change, particularly in the northern parts of their distributions. If land-use continues to change in more northern latitudes, this could limit the areas into which *Epipactis* can move in response to climate change. The results of our study provide further support for the potential for orchid ranges to increase at their leading edges in response to climate change, but go further to demonstrate that this does not necessarily mean an increase in available habitat, particularly if they cannot move into the northern habitats in time.

Testing the Range Size Vulnerability Hypothesis

A widely supported paradigm is that the maximum range limits of a species coincide with its ecological niche limits and that, given the opportunity to disperse, range limits will shift to match the geographical extent of the niche under climate change (Reed et al., 2021; Shay et al., 2021). The pattern of species with wider niche breadths demonstrating greater latitudinal shifts in response to climate change has been documented in a number of terrestrial plant taxa (Thuiller et al., 2005; Alarcón and Cavieres, 2018). This was also demonstrated in orchids by Geppert et al. (2020) where the distributions of generalist orchid species and those inhabiting forests and semi-natural grasslands tended to be less affected than the more specialised and rare species in subalpine, natural grassland and wetland habitats, whose rear and leading edges shifted upward. This corresponds with our finding that *Epipactis* species with wider niche breadths (generalists) experience greater change in habitat area in response to changing climate than specialists. If we were to assign species to the groups of specialist and generalist based on current spatial ranges and values of B2 in geographic space, *E. fageticola*, *albensis*, *kleinii*, and *lusitanica* would be considered the most specialist (relative to the other species in this study), followed by *E. tremolsii*, *dunensis*, and *leptochila* as moderately specialist (**Supplementary Table 2**). *E. muelleri*, *microphylla*, and *purpurata* are moderately generalist, while *E. helleborine*, *palustris*, and *atrorubens* could be considered generalists. However, *E. purpurata* had a low B2 value but a fairly large spatial distribution and *E. phyllanthes* a high B2 value and small range, which is in contrast to this pattern. *E. purpurata* was predicted to experience a significant decrease in suitable habitat under climate change which may indicate that species with relatively large current ranges may still have fairly narrow niches which are nonetheless currently common in the environment, but are under threat from changing climate.

The distributions of all investigated species, even generalists, tended to lag behind climate warming, without being able to fully track the upward shift in suitable climate resulting in a range contraction, in both our study and Geppert et al. (2020). Plant species inhabiting forests may be somewhat buffered from the effects of climate warming (De Frenne et al., 2013; Zellweger et al., 2020) and those in grasslands tend to have high thermal ranges because of the lack of this buffering (Geppert et al., 2020). Similarly, Vogt-Schilb et al. (2015) found higher rates of disappearances in wetland orchid species in Western Europe than those in grassland, and more appearances in forest. There did not seem to be any clear pattern in response to climate change and habitat-use in our species (other than with niche breadth), with woodland species such as *E. muelleri* decreasing in suitable habitat and *E. microphylla* increasing. However, our study used a broad-scale specification of land cover, while more may be revealed at a finer resolution that captures microclimate gradients.

An important caveat to consider when carrying out niche breadth studies is that the metric used to describe niche breadth can greatly affect the results. Levin's B2 is the reciprocal of Simpson's diversity index (Levins, 1968) and has been a popular metric of niche breadth for more than 50 years. However, it has been noted that the traditional calculation of this metric is in geographic space (see Peterson and Soberón, 2012) for more on geographic and environmental space) and more accurately represents the "flatness" of the geographic distribution of suitable habitat (Warren et al., 2019), which may be a useful measure of spatial habitat-use but is not niche breadth in terms of specificity of resource-use. This is demonstrated clearly in our results, where B2 in geographic space was consistently correlated with measures of the size of the habitat distribution and the mean Gibbs value. B2 in environmental space as proposed by Warren et al. (2019) and developed in Warren et al. (2021) filters the geographic habitat suitability distribution through the set of environmental variables that was used to create the Maxent model, resulting in a B2 value that is closer to the concept of niche breadth as being the specificity in environmental conditions of a species' habitat. It is important to note, however, that although closer to what we understand to be niche breadth, B2 in environmental space is still dependent on the availability of habitats in geographic space (Petraitis, 1979; Warren et al., 2019). Although the values of B2 in geographic and environmental space were not correlated, B2 in environmental space showed some evidence for having the same relationship with habitat changes as B2 in geographic space. This indicates that B2 in environmental space has the potential to be a useful representation of niche breadth for *Epipactis* in Europe, but further study is required to conclude this.

Other Factors Contributing to Range Shifts

Although the abiotic characteristics discussed here are important for predicting orchid ranges, biotic interactions and species-specific characteristics are also essential contributors to the realised niche, and including these interactions can improve the accuracy and performance of niche models

(Flores-Tolentino et al., 2020; Phillips et al., 2020). Orchids rely on insect pollinators and mycorrhizal fungi to reproduce and germinate (Rasmussen, 2008; McCormick and Jacquemyn, 2014). As with other pollinator-reliant plants, allogamous *Epipactis* will only persist and track shifting climate if their pollinators are also able to disperse (Benning and Moeller, 2019; Shay et al., 2021), such as has been predicted for a Neotropical orchid bee which is predicted to persist and increase its habitat range under future climate change (Silva et al., 2015). Mating system was not significantly associated with changes in habitat distribution in response to climate change, indicating that in the specific case of these species, autogamous and allogamous species did differ in response to predicted climate change. This is not surprising, considering that mating system was not significantly associated with niche breadth or range size for *Epipactis* species in previous studies (Evans and Jacquemyn, 2020) and niche breadth in geographic space is directly linked to range size. The presence of soil microbes has also been linked to the ability of plants to expand into newly available habitats (David et al., 2019; Bueno de Mesquita et al., 2020; Benning and Moeller, 2021; Shay et al., 2021). The diversity of mycorrhizal fungi is linked to latitudinal gradients for some orchid species (Duffy et al., 2019), but it is unclear whether the northern shifts in orchid distributions will be supported by the lower diversity of fungi in more northern latitudes. Our understanding and predictions of orchid distribution changes in response to climate change would be greatly improved with the addition of pollinator and fungal symbiont distributional data.

Implications for Conservation

Studies that model the ecological niches of species are useful for conservation planning, particularly for identifying newly accessible areas available to plants (more so than predicting range contractions) and assessing the risk faced by populations as a consequence of their range size (Schwartz, 2012; Shay et al., 2021). This study provides a useful estimate of new areas into which *Epipactis* can expand, which in conjunction with more information on predicted land change in these areas, could be used in conservation schemes to allow the genus to flourish under climate change. However, it is important to assess the results in light of individual patterns in addition to drawing general conclusions. This is demonstrated in the contrast between mean change in Gibbs value (no change) and the change in Gibbs value for individual species, where a number of species were predicted to increase in the future and, the increase in habitat area for some species and the decrease for other. This disparity between general vs. individual species patterns has also been demonstrated in Geppert et al. (2020), who showed high interspecific variation among orchids grouped by habitat preference. We also show how some species with large areas of habitat such as *E. purpurata* should not be considered immune to the detrimental effects of future climate change as they may suffer considerable range reductions if they are not able to sufficiently disperse northwards.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AE and HJ: conceptualisation, methodology, writing, review and editing, and funding acquisition. AE: formal analysis, data curation, and visualisation. HJ: supervision. Both authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.894616/full#supplementary-material

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Orchid diversity along an altitudinal gradient in the central Balkans

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Understanding patterns of species diversity along an altitudinal gradient is the major topic of much biogeographical and ecological research. The aim of this study was to explore how richness and density of orchid species and subspecies in terms of different categories of underground organ systems and pollination systems vary along an altitudinal gradient in the central Balkans. The altitudinal gradient of the study area was divided into 21 100-m vertical intervals. Data were analyzed using both non-linear and linear regressions with three data sets (total orchids, orchids of forest habitats, orchids of non-forest habitats) in the case of species richness and three data sets (total orchids—total area, forest orchids—forest area, and orchids of non-forest habitats—non-forest area) in the case of species density. The results showed a hump-shaped pattern of orchid richness and density, peaking at 900–1,000 m. The richness and density of orchids of forest habitats are generally slightly greater than the richness and density of orchids of non-forest habitats in lowland areas, whereas the orchids of herbaceous vegetation types dominating at high altitudes. Tuberous orchids dominate in low and mid-altitude areas, orchids with palmately lobed and fusiform tubers ("intermediate orchids") dominate at high altitudes, while rhizomatous orchids are predominate in mid-altitude forest stands. Both deceptive and self-pollinated orchids show a unimodal trend with a peak at mid-altitude areas. This study underlines the importance of low and mid-altitude areas for the survival of deceptive orchids and the importance of mid- and high-altitude areas for the survival of rewarding orchids. In addition, forest habitats at mid-altitudes have been shown to be crucial for the survival of self-pollinated orchids. The results suggest that the altitudinal patterns of orchid richness and density in the central Balkans are determined by mechanisms related to land area size and habitat cover, partially confirming the species-area relationship (SAR) hypothesis. This study contributes significantly to a better understanding of the potential impacts of habitat changes on orchid diversity, thereby facilitating more effective conservation planning.

KEYWORDS

Orchidaceae, ecology, altitudinal patterns, distribution, life history strategies, species richness, species diversity, Balkan Peninsula

Introduction

The family Orchidaceae is one of the most species-rich families in the plant kingdom, with an estimated 26,000–28,000 species from 749 genera (Christenhusz and Byng, 2016; Chase et al., 2017). Although orchids grow in almost all terrestrial ecosystems, they are most diverse in the tropics and subtropics, where species of different life forms can be found. In Europe, orchids are exclusively terrestrial, inhabiting both forest habitats and herbaceous plant communities (Djordjević and Tsiftsis, 2022). Because of their germination limitation, mycorrhizal specificity, and pollinator specialization, many orchid species are particularly vulnerable to environmental change (Waterman and Bidartondo, 2008; Swarts and Dixon, 2009). Intensive anthropogenic impacts resulting in habitat alteration and loss have led to the extinction or decline in abundance and distribution of many orchids (Kull and Hutchings, 2006). Understanding patterns of orchid species richness and abundance along the geographical and environmental gradients is a central goal of much ecological and biogeographical research (Tsiftsis et al., 2008; Acharya et al., 2011; Zhang et al., 2015a; Djordjević et al., 2016, 2020). In addition, knowledge of diversity patterns along the altitudinal gradient and factors influencing these patterns can contribute not only to a better understanding of orchid ecology and distribution, but also to planning strategies necessary for a successful species conservation plan.

In general, there are two main patterns of species richness—an altitude relationship: a monotonic decrease in number of species with altitude; and a hump-shaped pattern with the highest species number at mid-altitudes (Rahbek, 1995; McCain and Grytnes, 2010; Timsina et al., 2021). Nearly half of the studies showed that the hump-shaped patterns are the most common ones, whereas other studies suggested either a monotonic decrease or an increase in the number of species with altitude (McCain and Grytnes, 2010; Timsina et al., 2021). Although several hypotheses have been proposed to explain patterns of orchid diversity along the altitudinal gradient, most studies address the influence of climatic factors, then the mid-domain effect (MDE), while less attention has been paid to the species-area relationship (SAR). The climatic gradient hypothesis predicts that species richness peaks at a particular altitude where a combination of growing conditions is optimal for the species. According to Colwell and Lees (2000), most species live in mid-altitude areas due to the geometric limit of the species' range. This pattern, known as the "mid-domain effect" (MDE), results from random overlap of the altitudinal range of species (Colwell and Hurtt, 1994; Colwell et al., 2004; Dunn et al., 2007). The concept of a species-area relationship suggests that species richness varies depending on size of the area of a certain altitude range, i.e., that maximum species richness occurs in the altitudinal zones that cover the largest area (Acharya et al., 2011; Karger et al., 2011; Trigas et al., 2013).

There are some studies and books that provide detailed information on the altitudinal range of individual orchid species in Europe or specific countries, suggesting that the altitudinal range of the same species can vary considerably within the range of its distribution (Baumann et al., 2006; Delforge, 2006; Jersáková et al., 2015). To date, many studies provide important information on how altitudinal gradients affect orchid species richness, but most of them have been conducted for Asian (Acharya et al., 2011; Zhang et al., 2015a,b; Timsina et al., 2021), American (Cardelús et al., 2006; Ackerman et al., 2007; Štípková et al., 2016), and African countries (Jacquemyn et al., 2005b, 2007), while there are just few studies on orchid richness along the altitudinal gradient in Europe (Tsiftsis et al., 2019; Štípková et al., 2020, 2021). The species-area relationship (SAR) has been investigated for some countries in Asia (Acharya et al., 2011; Zhang et al., 2015a), whereas in Europe there are only two studies (Štípková et al., 2020, 2021) that consider this relationship by analysis of density. However, it has not been studied in detail how the area of specific habitats affects the patterns of orchid diversity along the altitudinal gradient.

In recent years, the diversity patterns of species classified in different functional groups have been used to understand the relationships between these traits and environmental variation (Laughlin et al., 2012; Taylor et al., 2021). There are several studies on the distribution of certain orchid life forms, including commonly terrestrial, epiphytic, and saprophytic orchids (Cardelús et al., 2006; Acharya et al., 2011; Zhang et al., 2015a), while knowledge on the distribution patterns of orchid life forms in Europe is limited (Tsiftsis et al., 2019; Štípková et al., 2021). Species diversity patterns related to specific life forms along gradients (e.g., altitude, latitude) not only may be useful from a basic ecology perspective, but they can also contribute to a better understanding of orchid evolutionary history, prediction of their distribution, and effective orchid species conservation. Some studies have focused particularly on the distribution and species richness of orchids possessing certain floral traits and breeding systems, as well as pollination systems (Arroyo et al., 1982; Jacquemyn et al., 2005b; Pellissier et al., 2010; Štípková et al., 2020). Although it was found that the relative occurrence of food-deceptive orchids decreases with increasing altitude in the territory of Switzerland (Pellissier et al., 2010), there is a lack of knowledge on how orchid diversity patterns vary when it comes to other orchid pollination systems, including rewarding, self-pollinated and other deceptive orchids. Furthermore, there is a lack of knowledge about the relationship between altitude and richness/density of orchids, which are characterized by different life forms and pollination systems in different habitats (e.g., forests and herbaceous plant communities) and regions.

Although the Balkan Peninsula is one of the parts of Europe with the highest number of orchid taxa (Djordjević et al., 2020), the patterns of species richness and density along the altitudinal gradient in the central Balkans have not yet been explored.

Therefore, our study aims to explore patterns of diversity of orchids along the altitudinal gradient in the central Balkans, with the goal of analyzing both the total orchid flora and the orchid flora of individual life forms and pollination systems. Special attention had to be paid to the analysis of the influence of the size of the area of individual altitudinal intervals on the patterns of diversity, focusing on the area of specific habitat types (forest and non-forest). Consequently, the importance of this study lies in the contribution to the knowledge of orchid life histories, ecology and distribution, but also in the creation of a good basis for more effective orchid conservation. We hypothesized that spatial patterns of forest and non-forest habitats along the altitudinal gradient affect orchid species diversity patterns. Moreover, we expected that orchids of different traits have different diversity patterns as well. Based on the evolutionary development of the underground organs of orchids (Averyanov, 1990; Tsiftsis et al., 2019), we assume that orchids with spheroid or ovoid tubers dominate at lower altitudes because they generally tolerate drought and warmer conditions best. On the other hand, orchids with palmately lobed and fusiform tubers are assumed to dominate at higher altitudes because their origin is related to the emergence of colder climates and they have the best adaptations that allow them to grow in habitats with low temperatures and high humidity characteristic of highland areas. Given the different pollination systems of orchids and the studies already published (Pellissier et al., 2010; Štípková et al., 2020), we expect that the richness of rewarding orchids is greater than that of deceptive ones in high-altitude areas.

The main objectives of this study were: (i) to determine altitudinal range size of individual orchids and compare altitudinal range size and mean altitude of occurrence of orchid species and subspecies with different life traits (underground organ systems, pollination systems); (ii) to analyze orchid species richness and density along the altitudinal gradient; (iii) to determine how the richness and density of orchid species with different life traits (underground organ systems, pollination systems) vary with altitude. Patterns of species richness and density along the altitudinal gradient were explored for the total orchid flora, as well as for the orchid flora recorded in forest and non-forest habitats.

Materials and methods

Study area

The study area covers the entire territory of western Serbia (19°09′-20°39′ E, 42°50′-44°58′ N) and encompasses approximately 18,000 km^2 (**Figure 1A**). It is located in the central Balkans and belongs to the eastern Dinaric Alps. Two basic units are distinguished in the study area: (a) the flatlands of the southern part of the Pannonian Plain, which occupy the northern parts of western Serbia, and (b) the mountainous region, which belongs to the Dinaric mountain system. The altitude ranges from 65 m (Šabac) to 2,154 m (Mokra Gora—Pogled). The climate in Serbia can be described as temperate-continental. The average annual temperature varies from 6.7°C in the coldest parts to 11.6°C in the warmest parts, while the average annual temperature in the areas above 1,500 m above sea level is about 3.0°C. Annual precipitation varies from 726.4 mm in the lower-lying regions to about 1,500 mm in the mountainous areas of south-western Serbia (climatic data from the Hydrometeorological Service of the Republic of Serbia).

In general, vegetation in the study area is structured according to climatic differentiation. In the northernmost parts of western Serbia, near the Sava and Kolubara rivers, there are floodplain (*Fraxino-Quercion roboris*) forests, while in the rest of the study area (especially at low to medium altitudes) oak (*Quercion confertae* and *Quercion petraeo-cerridis*) forests predominate. Mesophilous deciduous beech and hornbeam (*Fagion sylvaticae* and *Carpinion betuli*) forests are predominant in the zone of middle altitudes, while coniferous (*Vaccinio-Piceetea*) forests are found in the high-mountain regions. The density of forest cover in the study area is shown in **Figure 1B**. Western Serbia is geologically diverse, with a large occurrence of carbonate and ultramafic rocks and various types of silicate rocks (Djordjević and Tsiftsis, 2019).

Data collection

The total database consists of data on 55 orchid species and subspecies recorded at 3,580 sites (**Supplementary Table 1**). Data on 53 orchid species and subspecies from 2,610 sites were collected during field observations between 1995 and 2021. In addition, the dataset included published data on 48 species and subspecies from 683 sites and herbarium data on 44 taxa from 287 sites collected in the Herbarium of the University of Belgrade (BEOU) and the Herbarium of the Natural History Museum in Belgrade (BEO). The number of sampling sites for each altitudinal interval is shown in **Figure 2**. This number does not include the sites we visited and did not find any orchids there. Orchid taxa were identified according to Delforge (2006), while Djordjević et al. (2021) was used for nomenclature. During field surveys, geographic coordinates (longitude, latitude) and altitude were determined by a Garmin eTrex 30 hand-held GPS device in the WGS 84, while reliable data from published sources and herbarium collections were georeferenced using Ozi Explorer 3.95.4s software.

We studied each altitudinal interval with the same effort, number of days spent in the field, and mileage. The minimum distance between sites was 250 m (i.e., two populations found closer than 250 m from each other were considered as one site), except in the case when large differences in altitude and habitats of the studied places were observed. Due to the relatively small size of the area searched and the long duration of the study, we

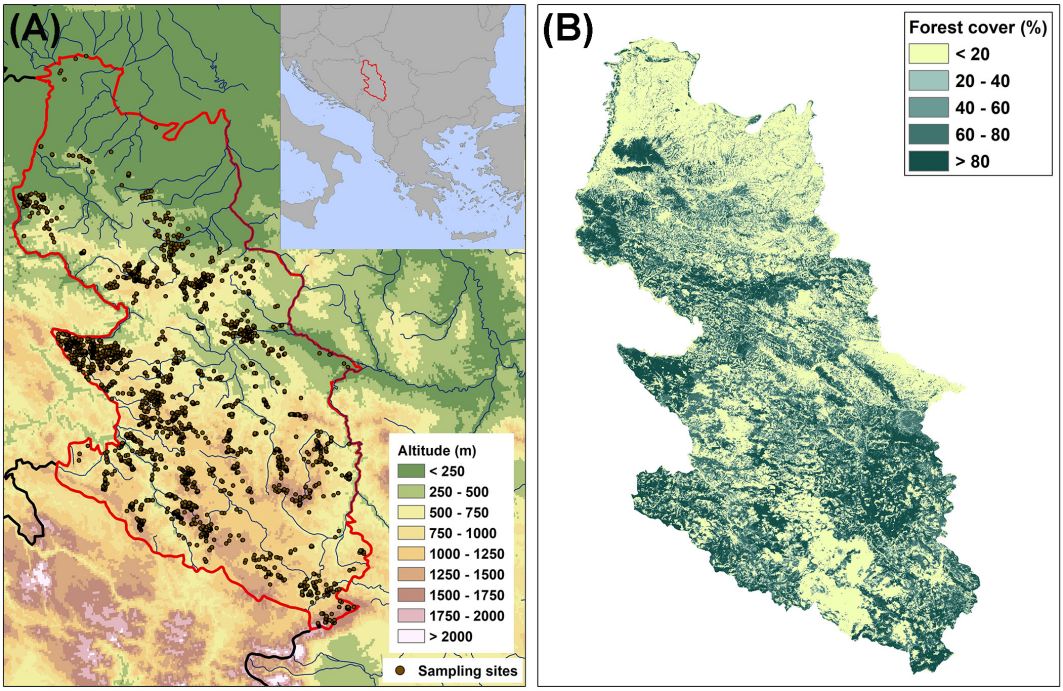

FIGURE 1
(A) Map of the study area (central Balkans: western Serbia) with sampling sites where orchids were found (the boundaries of the study area are marked by the red line); **(B)** the density of forest cover in the study area.

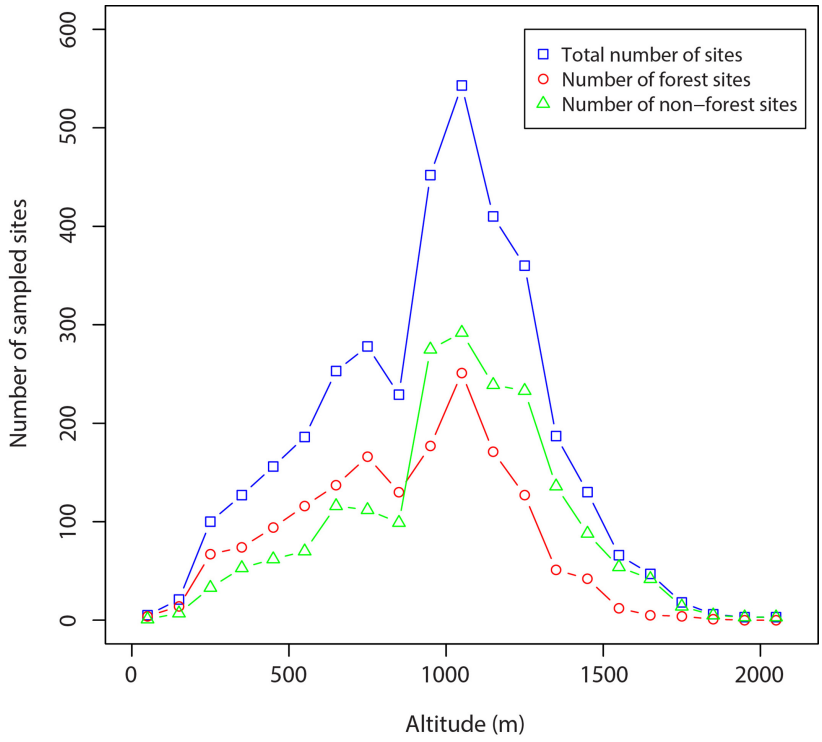

FIGURE 2
The number of sampling sites for each altitudinal interval where orchids were found.

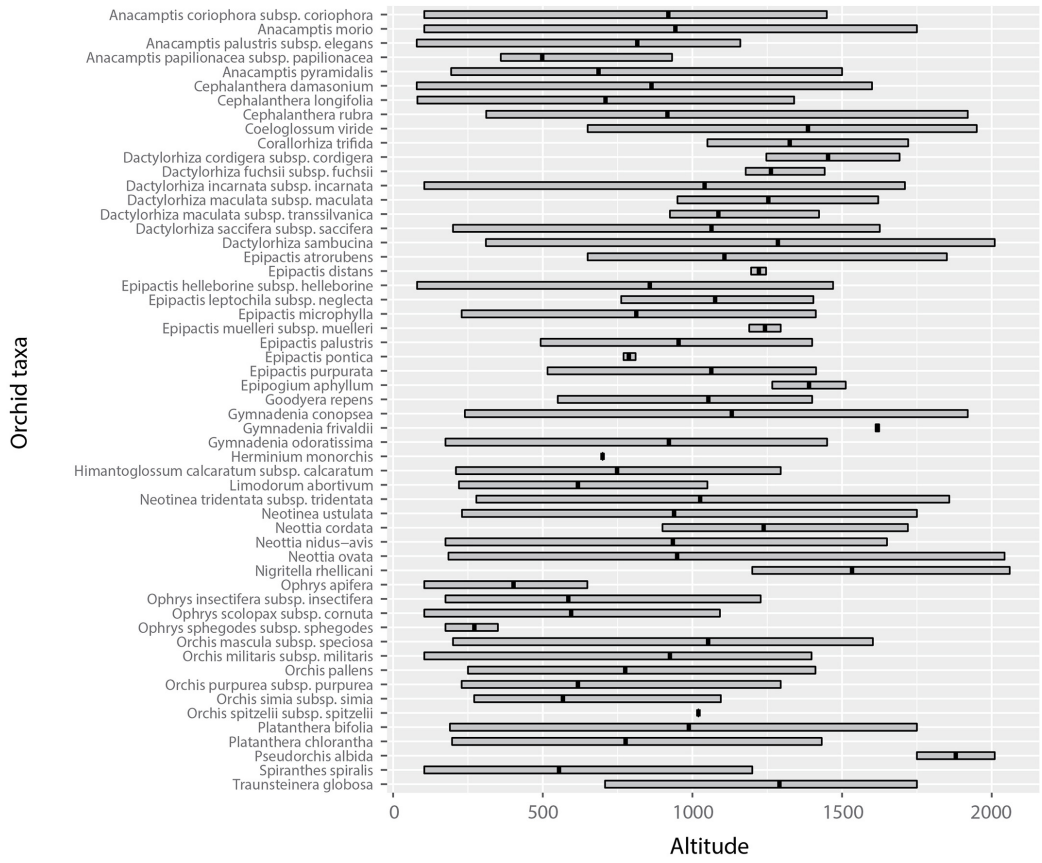

FIGURE 3
The altitudinal range of orchids in western Serbia (the central Balkans).

are sure that the number of sites we missed during the search is negligible and therefore cannot have affected the outcome. For the same reason, we can expect that the sampling effort was uniform throughout the region. For subsequent calculations, we only considered sites where orchids were found.

Subdivision of orchid species by habitat type, life forms (underground organs), and pollination systems

Orchid species were divided into two categories based on habitat type: (1) orchids that were recorded in forest habitats and (2) orchids that inhabit non-forest habitats (grasslands and herbaceous wetlands) (**Supplementary Table 1**). Orchids that occurred in both forest and non-forest habitats were included in both habitat categories (counted twice). In addition, orchids were relegated to various categories based on their underground organs and pollination systems (**Supplementary Table 1**). We classified orchids as belonging to one of three underground organ systems, following the concept presented by Tsiftsis et al. (2019) and Štípková et al. (2021): (1) rhizomatous orchids (the most primitive ones); (2) "intermediate orchids," i.e., orchids with palmate, fusiform, or stoloniferous tubers (intermediate in evolutionary history between rhizomatous orchids and orchids with spheroid tubers); and (3) tuberous orchids, i.e., orchids with spheroid tubers (considered the most specialized and advanced orchids). Species of the genera *Cephalanthera*, *Corallorhiza*, *Epipactis*, *Epipogium*, *Goodyera*, *Limodorum*, and *Neottia* were classified in the rhizomatous orchid group, while species of the genera *Coeloglossum*, *Dactylorhiza*, *Gymnadenia*, *Nigritella*, *Platanthera*, and *Pseudorchis* were placed in the intermediate group. In addition, species of the genera *Anacamptis*, *Herminium*, *Himantoglossum*, *Neotinea*, *Ophrys*, *Orchis*, *Spiranthes*, and *Traunsteinera* were classified as tuberous orchids.

Based on their pollination system, orchids were divided into three categories: (1) rewarding orchids, i.e., those that produce nectar and offer it as a reward to their pollinators, (2) deceptive, and (3) self-pollinated species (**Supplementary Table 1**). Information on the pollination mechanism of orchids was obtained from Jacquemyn et al. (2005a), Jersáková et al. (2006), Vereecken et al. (2010), and Inda et al. (2012), while for the genus *Epipactis* the AHO-Bayern webpage (Aho-Bayern, 2021) was used. Orchids that have nectar and thus could

TABLE 1 Overall statistics of the polynomial regressions used to determine the relationship between orchid species richness and altitude.

Orchid group	R^2	*P-value*
Total orchids	0.9267 (c)	<0.001
Orchids of forest habitats	0.9295 (c)	<0.001
Orchids of non-forest habitats	0.9061 (c)	<0.001

(c): 3rd order polynomial regression.

be rewarding but can also be self-pollinated (e.g., *Epipactis* spp.) were classified in both categories (rewarding and self-pollinated plants).

Data analysis

The altitudinal gradient of the study area was divided into twenty-one 100 m vertical intervals (i.e., 0–100 m, 101–200 m, etc.). Species richness was calculated for each 100-m altitudinal interval as the total number of orchid species and subspecies in that interval. The area (in km^2) of each 100-m interval was estimated by counting the number of 100-m grid cells of a Digital Altitude Model (DEM) having altitude values at a specific vertical interval. To achieve the 100-m map, the 25-m European Digital Altitude Model (Copernicus, EU-DEM version 1.1) was used by carrying out an aggregation process. The Tree Cover Density layer (2015 was used as the reference year) at a 100-m resolution (available through the Copernicus Land Monitoring Service) was used to calculate the forest area at each 100-m vertical interval, and then the non-forest area was calculated by removing the forested area from the total area in each vertical interval.

An orchid was considered as present in a 100-m interval only if it was recorded at least once in this vertical interval. After constructing the total matrix for all the orchid taxa recorded in the study area, two series of orchid matrices were generated according to the traits studied (underground organ system category, pollination system). Specifically, for each orchid category the number of orchid taxa occurring in each vertical interval was calculated.

To explore whether (a) the altitudinal range and (b) the mean altitude of occurrence of the orchids with different underground organ systems and pollination systems are statistically different, we used the Kruskal-Wallis test, followed by Dunn's *post-hoc* test with Bonferroni correction carried out on each pair of groups. The altitudinal range for each orchid was defined as the difference between the highest and the lowest site where each orchid has been recorded, whereas the mean altitude was calculated on the

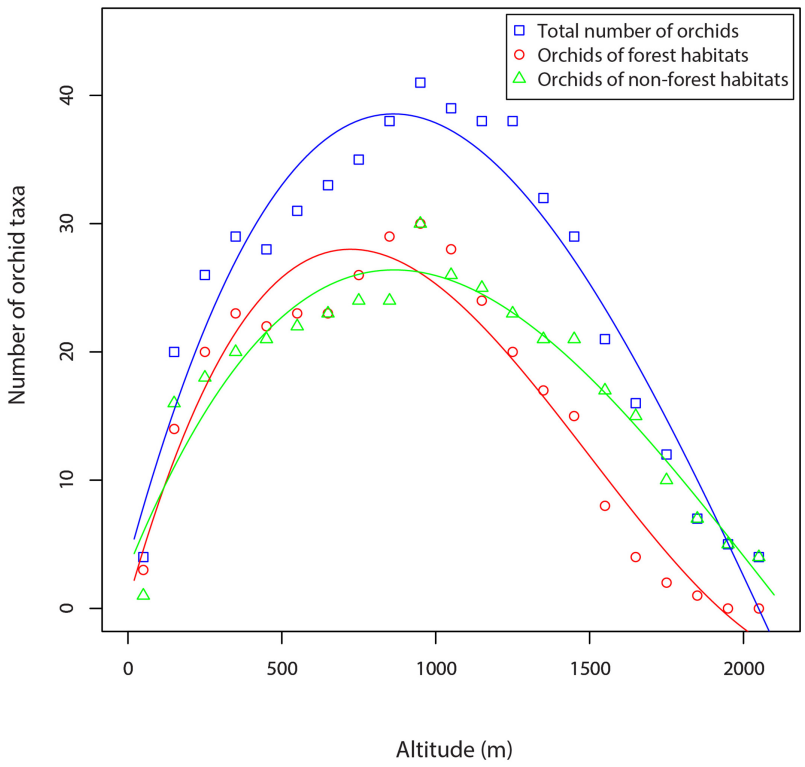

FIGURE 4
Orchid species richness along an altitudinal gradient in the central Balkans (western Serbia).

basis of all altitude values of the sites where each orchid has been recorded.

Orchid species density (D) at each altitude interval was calculated using the formula:

$$D = S/log(A),$$

where S is the number of orchid species recorded in each vertical interval and A is the area of each vertical interval in km^2.

Orchid density was calculated using (a) the total orchid flora and total area of the vertical intervals, (b) orchids of forest habitats and the forest area, and (c) orchids of non-forest habitats and the non-forest area of the vertical intervals. The three data sets were used to identify possible specific patterns in the orchids of these broad habitat categories.

The associations between richness and density of orchid species and subspecies and altitude were explored by analyzing the data sets using regressions. As we did not have any *a priori* hypothesis about the functions describing the shape of the dependences studied, polynomial regressions were used. We first used third-degree polynomials and always tested significance of the cubic terms in order to determine whether a second-degree or a linear regression would not be sufficient for fitting the data. Linear regression was used in cases where both cubic and quadratic terms were insignificant (Tsiftsis et al., 2019; Štípková et al., 2020, 2021).

All analyses were performed in R version 4.0.5 (R Core Team, 2021), whereas variable extraction was done using ArcGIS 10.6 (ESRI, 2017). Kruskal-Wallis and Dunn's *post-hoc* tests were performed using the packages "stats" and "FSA" (Ogle et al., 2022), respectively.

Results

Altitudinal range size

Altitudinal range profiles of orchid species and subspecies of the central Balkans (western Serbia) showed that most species occurred over wide altitudinal ranges (**Figure 3** and **Supplementary Table 1**). Thus, 12 species and subspecies (21.82%) had altitudinal ranges less than 500 m, 13 species and subspecies (23.64%) had altitudinal ranges from 500 to 1,000 m, 20 taxa (36.36%) had altitudinal ranges from 1,000 to 1,500 m, while 10 taxa (18.18%) had altitudinal ranges of more than 1,500 m (**Figure 3** and **Supplementary Table 1**).

There was no significant difference in altitudinal range between orchids with different types of underground organs (Kruskal-Wallis $\chi^2 = 0.314$, $p = 0.854$) or pollination systems ($\chi^2 = 1.635$, $p = 0.441$). In contrast, the mean altitude of occurrence differed significantly between orchid species with different underground organs ($\chi^2 = 21.617$, $p < 0.001$). In particular, intermediate orchids had a significantly higher mean altitude of occurrence than rhizomatous orchids ($Z = 2.01$, $p < 0.05$) and tuberous orchids ($Z = 4.589$, $p < 0.001$). Moreover, rhizomatous orchids had a significantly higher mean altitude than tuberous orchids ($Z = 2.707568$, $p < 0.01$). Similarly, the mean altitude of occurrence differed between orchid species with different pollination systems ($\chi^2 = 6.6227$, $p < 0.05$), with the mean altitude of occurrence of deceptive orchids

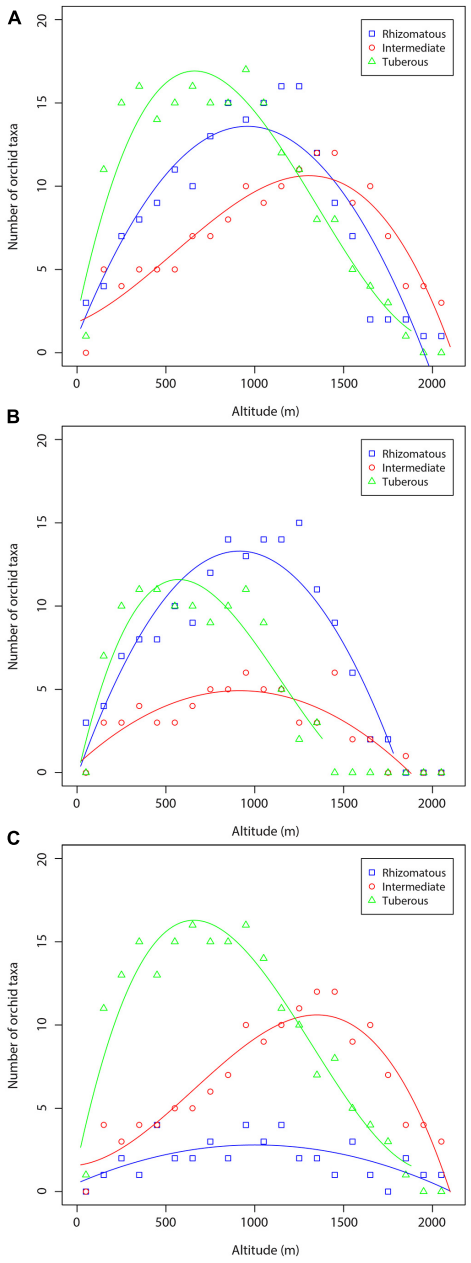

FIGURE 5
Richness of orchid species and subspecies with certain underground organ systems along the altitudinal gradient: **(A)** total number of orchid taxa; **(B)** orchids of forest habitats; **(C)** orchids of non-forest habitats.

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being significantly lower that of rewarding orchids ($Z = -2.573$, $p < 0.05$). No significant difference was found between the mean altitude of occurrence of deceptive and self-pollinated orchids ($Z = -0.861$, $p = 0.973$), and rewarding and self-pollinated orchids ($Z = 1.086$, $p = 0.832$).

Altitudinal patterns of orchid species richness

Regression analysis showed a strong influence of altitude on orchid species richness in western Serbia (Table 1 and Figure 4). The total orchid species richness showed a hump-shaped relationship along the altitudinal gradient. Species richness reached its maximum value in the mid-altitude zone between 901 and 1,000 m (41 orchid species and subspecies) and then decreased to reach its minimum at high-altitude sites (Table 1 and Figure 4).

Regression analysis showed a significant effect of altitude on orchid species richness in both forest and non-forest habitats (Table 1 and Figure 4). Both orchids of forest habitats and orchids of non-forest habitats showed a hump-shaped relationship with the altitudinal gradient, peaking between 901 and 1,000 m (Figure 4). In the altitudinal zone from 0 to 1,100 m, the richness of orchids of forest habitats is generally higher than the richness of orchids of non-forest habitats (Figure 4). However, with increasing altitude (from 1,101 to 2,100 m), the richness of orchids of non-forest habitats is higher than the richness of orchids of forest habitats (Figure 4).

Orchid richness in terms of the number of rhizomatous, intermediate, and tuberous orchid taxa for the three data sets (total orchids, orchids of forest habitats, orchids of non-forest habitats) are shown in Figure 5. The regression lines of orchids of each orchid life form have rather the same shape (a hump-shaped pattern). Tuberous species dominate at low and mid-altitude zone, the rhizomatous orchids present their highest richness at c. 1,100–1,300 m, whereas intermediate orchids dominate at high-altitude areas (Figure 5A). The results concerning orchids of the forest habitats were of the similar shape for all three orchid groups (Figure 5B). However, tuberous orchids dominate at low altitude areas, whereas the rhizomatous orchids dominate from mid-altitude area to high-altitude zone (Figure 5B). In the case of orchids of non-forest habitats, tuberous orchids dominate in low and mid-altitudinal zone (0–1,200 m), whereas the intermediate orchids dominate between 1,200 and 2,100 m (Figure 5C).

The trends in orchid species richness along the altitudinal gradient based on the three pollination mechanisms are shown in Figure 6. The orchids of each orchid pollination system showed a hump-shaped relationship with the altitudinal gradient, peaking at mid-altitude zone (Figure 6A). In general, the richness of deceptive orchids is greater than the richness of rewarding and self-pollinated orchids at altitudes between 0 and 1,700 m, whereas the richness of rewarding orchids is higher than the richness of deceptive and self-pollinated orchids at altitudes between 1,700 and 2,100 m (Figure 6A). The altitudinal patterns of orchid species richness of specific pollination systems were hump-shaped also in cases when orchids of forest and non-forest habitats were considered separately (Figures 6B,C). All the correlations between orchid species richness and altitude using the three datasets were statistically significant ($P < 0.001$

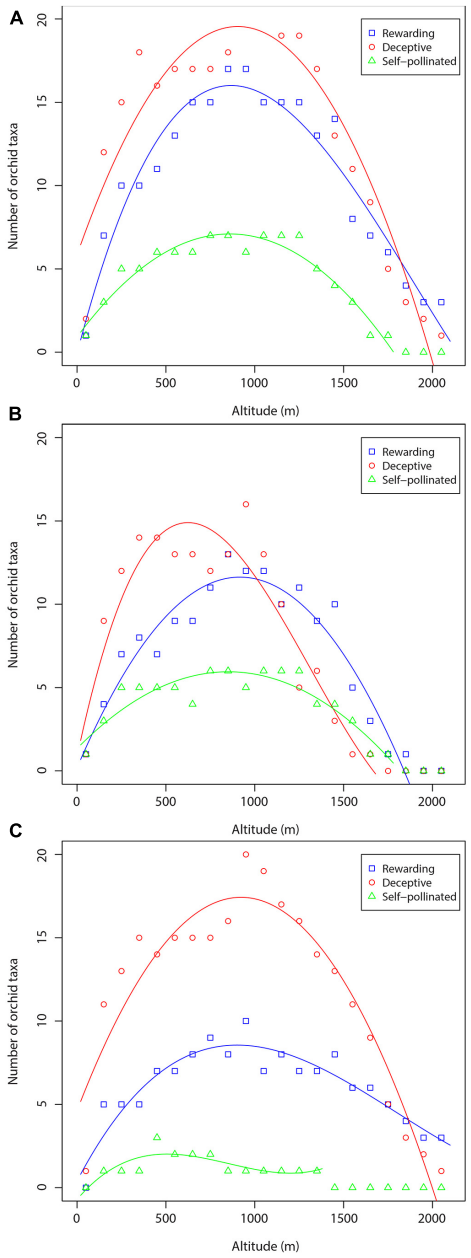

FIGURE 6
Richness of orchid species and subspecies of specific pollination systems along the altitudinal gradient: **(A)** total number of orchid taxa; **(B)** orchids of forest habitats; **(C)** orchids of non-forest habitats.

TABLE 2 Overall statistics of the polynomial regressions used to determine the relationship between the richness of orchid species with specific underground organ systems and altitude.

Orchid group	R^2	*P-value*
Total orchids		
Tuberous	0.906 (c)	<0.001
Rhizomatous	0.842 (b)	<0.001
Intermediate	0.829 (b)	<0.001
Orchids of forest habitats		
Tuberous	0.822 (c)	<0.001
Rhizomatous	0.861 (b)	<0.001
Intermediate	0.676 (b)	<0.001
Orchids of non-forest habitats		
Tuberous	0.923 (c)	<0.001
Rhizomatous	0.420 (b)	<0.01
Intermediate	0.885 (c)	<0.001

(b): 2nd order polynomial regression; (c): 3rd order polynomial regression.

TABLE 3 Overall statistics of the polynomial regressions used to determine the relationship between the richness of orchid species with specific pollination systems and altitude.

Orchid group	R^2	*P-value*
Total orchids		
Rewarding	0.938 (c)	<0.001
Deceptive	0.889 (b)	<0.001
Self-pollinated	0.924 (b)	<0.001
Orchids of forest habitats		
Rewarding	0.914 (b)	<0.001
Deceptive	0.882 (c)	<0.001
Self-pollinated	0.821 (b)	<0.001
Orchids of non-forest habitats		
Rewarding	0.869 (c)	<0.001
Deceptive	0.891 (b)	<0.001
Self-pollinated	0.664 (c)	<0.01

(b): 2nd order polynomial regression; (c): 3rd order polynomial regression.

or $P < 0.01$) (Tables 2, 3), whereas the predictive power was very high in almost all regressions.

Altitudinal patterns of orchid species density

Regression analysis showed a strong influence of altitude on orchid species density in the central Balkans (Table 4 and Figure 7). The regression lines of total orchids and orchids of forest habitats have rather the same shape (a hump-shaped pattern). Total species density reached its maximum value in the mid-altitude zone (at *c.* 1,000 m) and then decreased to reach its minimum in high-altitude areas (Figure 7). Species density of orchids of non-forest habitats increased with increase in altitude, peaking at about 800 m, then slightly decreased or

TABLE 4 Overall statistics of the polynomial regressions used to determine the relationship between orchid species density and altitude.

Orchid group	R^2	*P-value*
Total orchids	0.8967 (c)	<0.01
Orchids of forest habitats	0.938 (c)	<0.001
Orchids of non-forest habitats	0.7711 (c)	<0.001

(c): 3rd order polynomial regression.

stabilized and slightly increased up to the highest altitudes. In the in lowland areas, the density of orchids of forest habitats is generally higher than the density of orchids of non-forest habitats, whereas the density of orchids of non-forest habitats is higher than the density of orchids of forest habitats at mid- to high-altitude zone (Figure 7).

Orchid densities in terms of the number of rhizomatous, intermediate, and tuberous orchid taxa for the three data sets (total orchids—total area, forest orchids—forested area, non-forest orchids—non-forested area) are shown in Figure 8. When the orchid density was calculated based on the total orchid flora and the total area at each vertical interval, the curves of rhizomatous and tuberous orchids have rather the same shape (a hump-shaped pattern), but the number of tuberous species is slightly higher (Figure 8A). Contrary to these two species groups, the intermediate orchids show an increasing trend, and their density is gradually stabilized above 1,500 m. The results concerning orchids of the forest area were of the same shape for all three orchid groups (Figure 8B). In the case of orchid density calculated using non-forest orchids and the non-forest area, the intermediate orchids showed a sharp increase along the altitudinal gradient, whereas the tuberous orchids showed a hump-shaped pattern (Figure 8C). Rhizomatous orchids have the lowest species density compared to the other two orchid groups.

The trends in orchid species density along the altitudinal gradient based on the three pollination mechanisms are shown in Figure 9. In the case of orchid density calculated based on the total number of orchids and the total area, both deceptive and self-pollinated orchids show a unimodal trend with a peak at about 900–1,000 m, the deceptive orchids being the richest in terms of species (Figure 9A). On the contrary, density of rewarding orchids increases sharply up to 900 m and then slightly decreases. The graph of orchid density of the forest habitat types is presented in Figure 9B. Here, all orchid groups show a unimodal trend. When analyzing non-forest orchids using the non-forested area, we found that the deceptive orchids showed a unimodal trend, reaching a maximum density at c. 1,000 m, much higher than density of the other orchid groups (Figure 9C). The density of rewarding orchids increases with increase in altitude, peaking at about 500 m, then is stabilized or slightly decreases up to c. 1,400 m before increasing again up to the highest altitudes. Self-pollinated species are only poorly

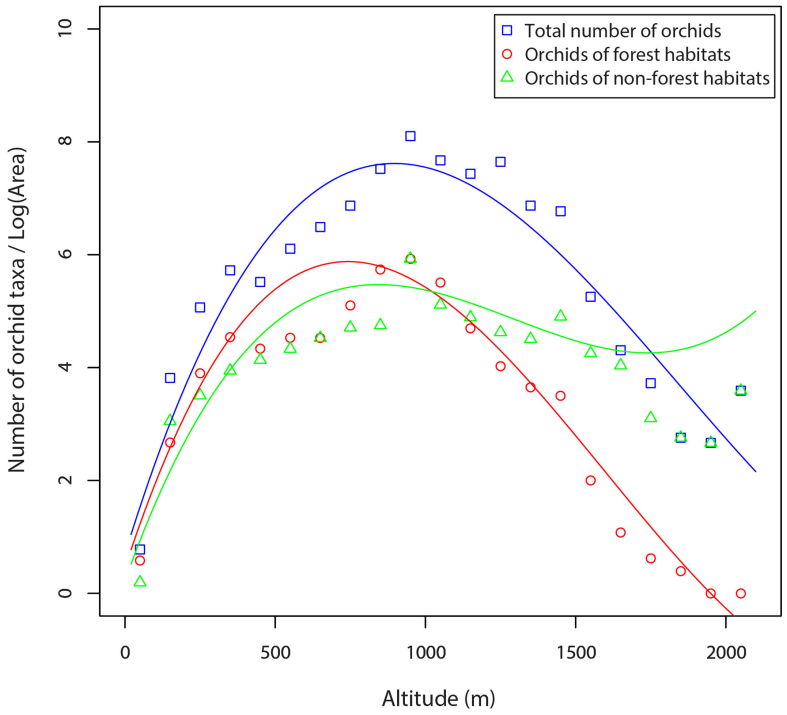

FIGURE 7
Orchid species density along an altitudinal gradient in the central Balkans (western Serbia).

represented in non-forested areas and show a slight hump-shaped pattern.

All the correlations between orchid species density and altitude using the three datasets were statistically significant ($P < 0.001$ or $P < 0.01$) (Tables 5, 6). Moreover, the predictive power was very high in almost all regressions. Specifically, the predictive power in the three datasets in the analyses performed using the underground organ system categories was 51.4–92.5%, whereas when analyzing orchids on the basis of their pollination mechanisms the predictive power of the regressions was 77.5–93.1%.

Discussion

In this study we investigated how the richness and density of orchids vary along the altitudinal gradient in the central Balkans. Specifically, we explored whether the forest and non-forest areas along the altitudinal gradient affect patterns of orchids richness and density using different functional traits. Our results showed a hump-shaped pattern of orchid richness and density, peaking in the mid-altitude area. In addition, the richness and density of orchids of forest habitats are generally slightly higher than the richness and density of orchids of non-forest habitats in lowland areas, while orchids of non-forest habitats dominate in high-altitude areas. The results showed that the diversity patterns of orchid species with different underground organs and pollination systems differ significantly along the altitudinal gradient when the orchid flora of specific habitat types was analyzed.

Altitudinal range size

The results of this study show that 10 orchid taxa have the largest altitudinal ranges (above 1,500 m) in the central Balkans (Figure 3 and Supplementary Table 1), highlighting their ecological plasticity and adaptability, as well as a lower degree of specialization.

Our results show that orchids belonging to the Central European and boreal chorological groups (*Coeloglossum viride*, *Dactylorhiza fuchsii*, *Goodyera repens*, *Epipactis leptochila* subsp. *neglecta*, *Epipactis muelleri*, *Epipactis purpurata*, *Epipogium aphyllum*, and *Neottia cordata*) occur in the middle and high altitudes of the central Balkans. In contrast, in Central and Northern Europe, these species have higher altitude ranges, from lowlands to high-mountain areas (Baumann et al., 2006; Delforge, 2006). In addition, in the central Balkans, orchids characteristic primarily of Central and Northern Europe have a greater elevational range or occur at lower altitudes compared to northeastern Greece (Tsiftsis et al., 2008). Furthermore, some Mediterranean-submediterranean orchids (e.g., *Anacamptis papilionacea*, *Neotinea tridentata*, and *Orchis simia*) have a lower altitudinal range and occur mainly at lower altitudes in

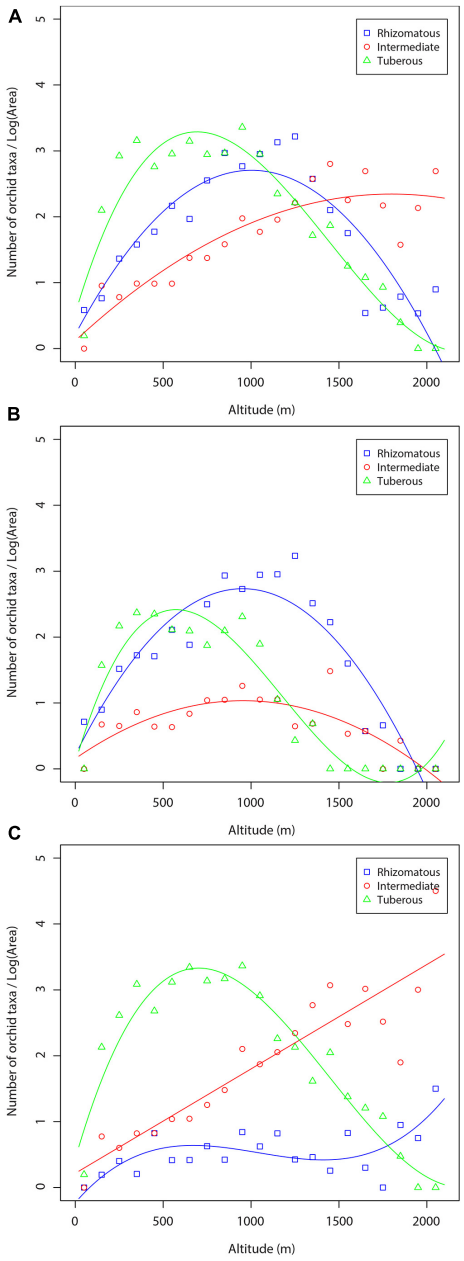

FIGURE 8
Density of orchid species and subspecies with certain underground organ systems along the altitudinal gradient: **(A)** total number of orchid taxa—total area; **(B)** forest orchids—forest area; **(C)** non-forest orchids—non-forest area.

western Serbia than in northeastern Greece (Tsiftsis et al., 2008), which can be explained primarily by the climatic differences between these two study areas. Indeed, north-eastern Greece is under strong influence of the Mediterranean climate and has a significant presence of thermophilous habitats along the altitudinal gradient. In contrast, in the central Balkans (western Serbia), due to the humid and continental climate, thermophilous habitats are mainly present at lower and middle altitudes.

Altitudinal patterns of orchid species richness and density

The results of this study show hump-shaped patterns of orchid richness and density along the altitudinal gradient in western Serbia, both in the case of total orchids and in the cases of orchids of specific habitat types. In all cases, the highest species richness and density were observed between 500 and 1,200 m. This is consistent with previous studies indicating that orchid species richness is highest at mid-altitudes and decreases with increasing altitude (Acharya et al., 2011; Chen et al., 2014; Liu et al., 2015; Zhang et al., 2015a). It is assumed that patterns of orchid species richness and density along the altitudinal gradient in the central Balkans (western Serbia) are primarily determined by climatic factors and breadth of the climatic niche of species composing the orchid pool in western Serbia. The highest orchid species richness and density at mid-altitudes in the central Balkans can be explained by the fact that most species tolerate the moderate environmental conditions in the middle altitudes better than the extreme environmental conditions, in terms of temperature, precipitation, relative air humidity, ultraviolet radiation, atmospheric pressure, partial pressure of all atmospheric gases, and anthropogenic influences in the low and high-altitudes (Lomolino, 2001; van der Meulen et al., 2001; Körner, 2007). The hump-shaped patterns of orchid richness and density along the altitudinal gradient can also be explained by size of the area. In western Serbia, area of the high-altitude zones (from 1,500 to 2,100 m) is rather restricted compared to areas at middle altitudes. On the contrary, although low-altitude areas (e.g., <500 m) are extensive in the study area, species richness and density in such areas are quite low because a large part of these areas has been converted to cultivated land and the landscape is not very heterogeneous in terms of habitats and geological substrates. Similarly, previous research has indicated that habitat heterogeneity overrides the species-area relationship and is the most important predictor of species richness (Báldi, 2008; Tsiftsis, 2020). In addition, it is assumed that the lower richness and density of orchids of non-forest habitats at lower altitudes can be explained by the intense anthropogenic influences. In general, the species richness of a given altitudinal range is related to its extent. However, this is correct for orchids of forest habitats, but not for orchids of non-forest habitats. Thus, our study partially confirms the SAR hypothesis (Karger et al., 2011). We could assume that the lower richness of orchid species in the high-altitude areas of western Serbia is determined by the lower diversity of their pollinators (Arroyo et al., 1982; Jacquemyn et al., 2005b), as well as by a smaller pollen load of pollinators (Bingham and Orthner, 1998). Furthermore, the greater richness and density of orchid species

of forest habitat types compared to the species richness and density of orchids of non-forest habitats in lowland areas can be explained primarily by the high diversity of forest communities in this altitude range. On the other hand, the greater richness and density of orchid species of herbaceous vegetation in relation to the richness and density of orchid species of forest vegetation in the high-altitude regions of western Serbia can be explained by the great diversity and high heterogeneity of grasslands and herbaceous wetlands, as well as by the lower diversity of forest vegetation.

Orchid species richness and density in terms of different underground organ systems

The results of this study show that tuberous orchids dominate in areas of lower and middle altitudes (**Figures 5**, **8**). This result was expected, bearing in mind that orchids of this life form are best adapted to dry, semi-dry, and warm habitat conditions, such as those found at the lower and middle altitudes of the study area (Dafni, 1987; Averyanov, 1990; Tsiftsis et al., 2019; Štípková et al., 2021). Rhizomatous orchids are predominant in mid-altitude areas, indicating that moderate environmental conditions are appropriate for them. However, the results showed that altitude strongly influences rhizomatous orchid species richness and density in forest habitats, whereas the influence of altitude is relatively weak when it comes to the richness and density of rhizomatous orchids in non-forest habitats. This can be explained by the fact that representatives of the orchids of this life form, as the most primitive representatives of European orchids, primarily grow in forest habitats (Averyanov, 1990; Delforge, 2006).

This study shows that intermediate orchids dominate in high-altitude areas, which is consistent with previous studies suggesting that these orchids prefer lower temperatures and higher humidity in their habitats and therefore occur in high-altitude areas (Averyanov, 1990; Delforge, 2006; Pillon et al., 2006; Tsiftsis et al., 2019). The results are understandable, bearing in mind the evolutionary development of orchids. Specifically, the evolution of the first intermediate orchids was associated with the Alpine orogenesis, and the formation of mountain habitats with lower temperatures (Averyanov, 1990).

Orchid species richness and density in terms of different pollination systems

The results of this study show that the richness and density of deceptive orchids are higher through almost all the altitudinal gradient studied, and that only in the highest regions of the investigated area do rewarding orchids prevail (**Figures 6**, **9**). This result is consistent with those of Pellissier et al. (2010), who found that the relative occurrence of food-deceptive orchids decreases with increasing altitude in the territory of Switzerland and in the Vaud mountains, suggesting that deception may be less profitable at high compared to low altitudes. This may be explained by climatic factors expressed through altitude, such as temperature, precipitation, or seasonality (Körner, 2007), as well as by factors that influence the decrease of pollinator diversity and visitation rate at high altitudes (Arroyo et al., 1982; Jacquemyn et al., 2005b; Pellissier et al., 2010).

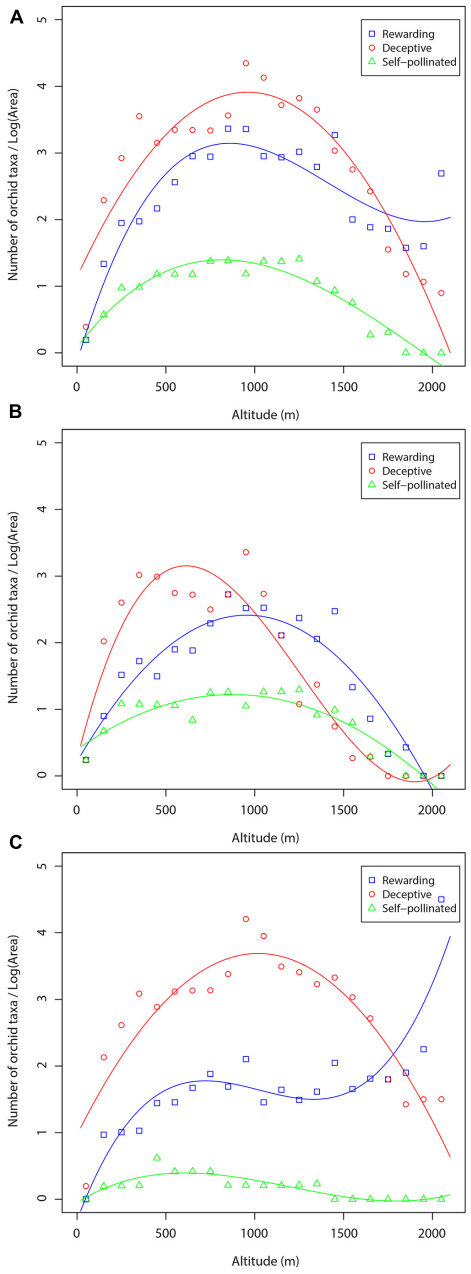

FIGURE 9
Density of orchid species and subspecies of specific pollination systems along the altitudinal gradient: **(A)** total number of orchid taxa—total area; **(B)** forest orchids—forest area; **(C)** non-forest orchids—non-forest area.

Štípková et al. (2020) used nectarless and nectariferous orchids of the Czech Republic and found that both groups showed a hump-shaped pattern of species density, with a maximum between 300 and 900 m, i.e., at lower altitudes

TABLE 5 Overall statistics of the polynomial regressions used to determine the relationship between the density of orchid species with specific underground organ systems and altitude.

Orchid group	R^2	*P-value*
Total orchids		
Tuberous	0.919 (c)	<0.001
Rhizomatous	0.789 (b)	<0.001
Intermediate	0.829 (b)	<0.001
Orchids of forest habitats		
Tuberous	0.912 (c)	<0.001
Rhizomatous	0.874 (b)	<0.001
Intermediate	0.667 (b)	<0.001
Orchids of non-forest habitats		
Tuberous	0.925 (c)	<0.001
Rhizomatous	0.514 (c)	<0.01
Intermediate	0.831 (a)	<0.001

(a): 1st order polynomial regression; (b): 2nd order polynomial regression; (c): 3rd order polynomial regression.

TABLE 6 Overall statistics of the polynomial regressions used to determine the relationship between the density of orchid species with specific pollination systems and altitude.

Orchid group	R^2	*P-value*
Total orchids		
Rewarding	0.822 (c)	<0.001
Deceptive	0.867 (b)	<0.001
Self-pollinated	0.931 (c)	<0.001
Orchids of forest habitats		
Rewarding	0.893 (b)	<0.001
Deceptive	0.920 (c)	<0.001
Self-pollinated	0.857 (b)	<0.001
Orchids of non-forest habitats		
Rewarding	0.807 (c)	<0.001
Deceptive	0.826 (b)	<0.001
Self-pollinated	0.775 (c)	<0.001

(b): 2nd order polynomial regression; (c): 3rd order polynomial regression.

compared to orchids in the central Balkans. Similarly to our results, species density of both nectariferous and nectarless orchids along the altitudinal gradient in the Czech Republic was found to depend on habitat cover, i.e., the spatial distribution of forest and non-forest habitats. Earlier studies of orchids have shown that most self-pollinated orchids occur in high-altitude areas (Catling, 1990; Jacquemyn et al., 2005b). Self-pollinated orchids in the central Balkans mostly inhabit forest vegetation types, so the density of these orchids is highest in mid-altitude areas, in which forest orchids dominate.

Implications for conservation

This study shows that forest and non-forest habitats at low and mid- altitudes have high conservation value for tuberous orchids, while forest habitats at mid-altitudes are important for the survival of rhizomatous orchids. In addition, non-forest habitats at mid- and high-altitudes are most important for the survival of intermediate orchids. Given the resulting diversity patterns and the fact that intermediate orchids inhabit colder and higher precipitation areas (Tsiftsis et al., 2019; Štípková et al., 2021), our study suggests that these orchids may be affected by the rise of temperature and lower precipitation at lower altitudes due to climate change.

Our study suggests that forest and non-forest habitats at low and mid-altitudes are most important for the survival of deceptive orchids. On the other hand, mid- and high-altitudinal areas are important for the survival of rewarding orchids. Since rewarding orchids are rarer at lower altitudes, they are at high risk of extinction in these areas. In view of the fact that the rewarding orchids in western Serbia occur in almost equal numbers in forest and non-forest habitats, it is necessary to carefully plan their conservation. Deceptive orchids in the central Balkans occur in slightly higher numbers in non-forest habitats (grasslands and meadows), a circumstance that requires careful conservation of these habitats. Finally, our study indicates that most orchid species grow in mid-altitude areas, which coincide with the strong presence of tourist sites and facilities in the study area. It is therefore necessary to work on a carefully designed plan for protection of these areas, including the application of ecologically sustainable tourism that does not threaten orchids to extinction.

Conclusion

This study demonstrates a hump-shaped pattern of orchid richness and density peaking at 900–1,000 m and the fact that orchid species diversity patterns differ significantly along the altitudinal gradient when comparing forest vs. non-forest habitats. In general, our results confirm the SAR hypothesis, i.e., that the richness and density of orchid species along the altitudinal gradient are significantly affected by size of the area of a given altitudinal interval. However, this does not hold true for the orchids of non-forest habitats. Furthermore, it does not hold true for the intermediate orchids in non-forest habitats or for the rewarding orchids in the same habitats because the species density of these groups increases with altitude.

Our study suggests that the diversity patterns of orchid species with different underground organs and pollination systems differ significantly along the altitudinal gradient when considering the total flora in the whole area, but also when analyzing the orchid flora of specific habitat types. In general, tuberous orchids dominate in low and mid-altitude areas, intermediate orchids dominate at high altitudes, while rhizomatous orchids are predominate in mid-altitude forest stands. This confirms the hypothesis of evolutionary development of orchids with different underground organs and their specific ecological requirements (Averyanov, 1990;

Tsiftsis et al., 2019; Štípková et al., 2021). Our study highlights the importance of low and mid-altitude areas for the survival of deceptive orchids and the importance of mid- and high-altitude areas for the survival of rewarding orchids. In addition, forest habitats at mid-altitudes have been shown to be crucial for the survival of self-pollinated orchids.

In general, our study shows that the strategies required to protect orchids change along the altitudinal gradient and depend on both functional traits of species and habitat cover. In addition, our results suggest that changes in habitat cover may be reflected in patterns of orchid diversity along the altitudinal gradient. Future research should reveal which climatic and other environmental factors are crucial for the changes in orchid species richness and density along the altitudinal gradient in the central Balkans.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Author contributions

VD: fieldwork. VD and ST: methodology and formal analysis. All authors conceptualization, writing – review and editing and read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Dominant *Dendrobium officinale* mycorrhizal partners vary among habitats and strongly induce seed germination *in vitro*

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Dendrobium officinale (Orchidaceae) is an endangered epiphytic orchid that has been well studied as a medicinal plant. Although previous studies have shown that various fungal isolates promote *D. officinale* seed germination and seedling development *in vitro*, mycorrhizal associations among its wild populations remain poorly understood. In this study, we identified mycorrhizal fungi associated with *D. officinale* (36 individuals from six sites) using Sanger sequencing and compared fungal communities among sites and habitats (lithophytic vs. epiphytic individuals). Among the obtained sequences, 76 belonged to orchid mycorrhizal fungi (OMF), among which Tulasnellaceae accounted for 45.8% and Serendipitaceae for 28.1%. The Serendipitaceae operational taxonomic unit (OTU) SE1 was the most dominant partner, accounting for 27.1% of all detected fungal sequences, followed by a Tulasnellaceae OTU, TU27, which accounted for 15.6%. The relative frequencies of Serendipitaceae and Tulasnellaceae differed greatly between lithophytic and epiphytic individuals. Serendipitaceae accounted for 47.3% of the OMF sequences among lithophytes, and Tulasnellaceae for 95.2% among epiphytes. Mycorrhizal community composition also varied among sites. We further conducted *in vitro* symbiotic culture from seeds with six fungal isolates. Two Serendipitaceae and two Tulasnellaceae isolates, including SE1 and TU27, significantly promoted seed germination and seedling development. These results indicate that *D. officinale* is mainly associated with Tulasnellaceae and Serendipitaceae as its main fungal partners, which strongly induced seed germination and seedling development *in vitro*, suggesting their association with *D. officinale* through its life cycle.

KEYWORDS

lithophytes, orchid, Serendipitaceae, Tulasnellaceae, wild populations, epiphytes

Introduction

Orchidaceae is among the largest angiosperm plant families, comprising more than 28,000 species (Christenhusz and Byng, 2016), 69% of which are epiphytic (Zotz, 2013). Orchids form symbiotic associations with mycorrhizal fungi, in which fungal hyphae penetrate living plant cells to form intracellular pelotons (Smith and Read, 2008). Orchid seeds are highly dependent on mycorrhizal fungi for carbon, nitrogen, and other nutrients during seed germination; such associations generally persist in mature plants (Rasmussen and Rasmussen, 2009). Most orchid mycorrhizal fungi (OMF) belong to a rhizoctonia aggregate, a polyphyletic group of fungi belonging to a combination of Tulasnellaceae, Serendipitaceae, and Ceratobasidiaceae (Rasmussen, 2002; Dearnaley et al., 2012). Orchid mycorrhizal associations do not always remain stable throughout the plant life cycle, with some orchids continuing their association with the same fungi and others switching partners from the seed germination to adult stages (Ventre Lespiaucq et al., 2021). Habitat type, which can be terrestrial, epiphytic, or lithophytic, also often affects mycorrhizal communities (Xing et al., 2019; Qin et al., 2020). OMF may have a significant impact on the distribution, abundance, and population dynamics of orchid species (Jacquemyn et al., 2012; McCormick et al., 2018). However, despite the rich diversity of epiphytic orchids, far fewer studies have explored OMF associations among epiphytic orchids than among terrestrial orchids.

The genus *Dendrobium* Swartz is among the largest genera in Orchidaceae, including approximately 1,450 species distributed in tropical and subtropical regions from India to Southeast Asia, China, Japan, and Oceania (Schuiteman, 2014). *Dendrobium* species have long been studied for their economic, medicinal, and ornamental value (Teixeira da Silva et al., 2015; Teoh, 2016). Mycorrhizal associations with *Dendrobium* species have also been investigated for the propagation of medicinal species and conservation of endangered species (Chen et al., 2021). However, most such studies have focused on symbiotic culture with fungal isolates from roots, seeds, or seedlings (Nontachaiyapoom et al., 2011; Mala et al., 2017; Maharjan et al., 2020), whereas mycorrhizal associations among wild orchid populations remain poorly understood, although a few studies have revealed *in situ* associations with several wild populations (Xing et al., 2013; Rammitsu et al., 2021).

Dendrobium officinale Kimura and Migo (syn. *Dendrobium stricklandianum* Rchb.f and *Dendrobium tosaense* Makino; Jin and Huang, 2015) is a component of many traditional Chinese medicines and its symbionts have been well studied (Ding et al., 2008; Jin et al., 2017; Zuo et al., 2021). This species is distributed from southern China to southern Japan, where it grows on cliffs (lithophyte) or tree trunks (epiphyte) covered with humus and moss (Zhu et al., 2009; Hou et al., 2012). Although various fungal isolates from *Dendrobium* species promote seed germination and seedling development in *D. officinale* (Guo and Xu, 1991; Wu et al., 2012; Shao et al., 2019; Wang et al., 2021), the mycorrhizal associations of its wild populations remain unclear. Mycorrhizal fungi can vary among sites and substrates (lithophytic or epiphytic individuals). *D. officinale* is an endangered species due to over collection; therefore, understanding the mycorrhizal associations of its wild populations is important for its conservation.

In this study, we also examined the effects of major and minor mycorrhizal fungal associations on seed germination, protocorm formation, and seedling development. We examined 36 wild *D. officinale* individuals (27 lithophytic and 9 epiphytic) sampled from six sites and conducted *in vitro* symbiotic seed germination testing using *D. officinale* seeds and six fungal isolates obtained from roots.

Materials and methods

Sample collection

In all, 36 *D. officinale* individuals were collected from six sites in Kochi and Kagoshima Prefectures in Japan (**Table 1**). To examine differences in mycorrhizal fungal associations between epiphytic and lithophytic individuals, we collected epiphytic root samples from seven tree species and lithophytic root samples from rocks, cement bridges, and a roof. Root samples (3–5 cm per plant) were washed with tap water, and hand-sliced sections were observed under a microscope to assess fungal colonization. Mycorrhizal root segments were cut into 1–2 cm fragments and stored in Tris-EDTA (TE) buffer at −20°C for fungal molecular identification. Sections with living hyphal coils were used for fungal isolation.

Fungal isolation

Root sections with living hyphal coils were washed with sterile distilled water (SDW) to remove bark debris from the root surface and crushed with forceps to disperse the viable hyphae coils into 100 mL SDW. Hyphal coils (pelotons) were collected using a micropipette and rinsed four times in sterile water. For culture, these pelotons with 20–40 μL SDW were dropped onto 1.5% agar medium containing 50 ppm streptomycin and tetracycline. Plates were incubated at 25 ± 1°C for 1 week. Fungal colonies that formed from single pelotons were transferred to fresh potato dextrose agar (PDA) plates for subculture. The fungal isolates obtained in this study were deposited in the Biological Resource Center of the National Institute of Technology and Evaluation (NBRC) (**Table 2**).

Molecular identification of mycorrhizal fungi

DNA was extracted from root samples as described previously (Rammitsu et al., 2021). Samples were crushed with forceps to disperse hyphal coils into TE buffer. We collected 100–200 coils per fragment and homogenized these with 20 μL TE buffer using a BioMasher II homogenizer (Nippi Inc., Tokyo, Japan). For fungal isolate DNA, hyphae growing on the culture medium were collected using a sterilized toothpick and suspended in 50 μL TE buffer. DNA was extracted from the suspension as described previously (Izumitsu et al., 2012). Polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) sequences was performed using the fungal universal primer pairs ITS1F/ITS4 (White et al., 1990; Gardes and Bruns, 1993) and ITS1F/ITS4B (Gardes and Bruns, 1993). These primer pairs failed to amplify sequences of Tulasnellaceae, which is a dominant mycorrhizal fungal family associated with orchids. Therefore, we also used the Tulasnellaceae-specific primer pairs ITS5/ITS4-Tul2 (White et al., 1990; Oja et al., 2015) and 5.8S-Tulngs/ITS4-Tul2 (Oja et al., 2015; Rammitsu et al., 2021). PCR amplification was

TABLE 1 Details of *Dendrobium officinale* and mycorrhizal samples used in this study.

Locality	Site no.	Habitat[a]	Substrate	Total no. of individuals	Roots			Isolates			Total OMF
					No. of individuals	No. of samples	OMF	No. of individuals	No. of isolates	OMF	
Kami-shi, Kochi Prefecture, Japan	S1	L	Cement block wall	3	3	8	8	2	4	4	12
		E	*Aesculus turbinata*	1	1	3	1	0	0	0	1
Yakushima-cho, Kagoshima	S2	L	Cement bridge	5	5	9	9	0	0	0	9
Prefecture, Japan		E	*Distylium racemosum*	1	1	1	0	0	0	0	0
		E	*Castanopsis cuspidata*	1	1	6	2	0	0	0	2
		E	*Glochidion obovatum*	1	1	2	2	0	0	0	2
		L	Cement bridge	9	1	1	1	9	15	12	13
	S3	L	Rock wall	5	3	3	3	2	8	7	10
	S4	L	Cement bridge	4	4	11	8	3	3	2	10
		E	*Athruphyllum neriifolium*	1	1	2	2	0	0	0	2
		L	Cement roof	1	1	3	1	0	0	0	1
		E	*Quercus salicina*	2	1	5	5	1	2	2	7
	S5	E	Unknown fallen tree	1	1	6	6	0	0	0	6
	S6	E	*Ficus superba*	1	0	0	0	1	2	1	1

[a]L, Lithophyte; E, Epiphyte.

TABLE 2 Fungal isolates from *Dendrobium officinale* used for symbiotic culture.

Family	Fungal OTU	Isolate ID	Site no.	Substrate	DDBJ accession no.	NBRC accession no.
Tulasnellaceae	TU10	F205	S1	Cement block wall	LC597350	NBRC 114085
	TU22	F868	S2	Cement bridge	LC683200	NBRC 115276
	TU27	F763	S4	Cement bridge	LC683202	NBRC 115262
Serendipitaceae	SE1	F809	S4	Cement bridge	LC683203	NBRC 115270
	SE5	F859	S6	*Ficus superba*	LC683204	NBRC 115275
Ceratobasidiaceae	CE18	F356	S3	Rock wall	LC597346	NBRC 114326

performed using MightyAmp DNA polymerase Ver.3 (TaKaRa, Shiga, Japan) in a total volume of 10 μL, containing 1 μL sample DNA, 5 μL 2 × MightyAmp buffer, 5 pmol each primer, 0.2 μL MightyAmp DNA Polymerase Ver.3, and 1 μL 10 × Additive for High Specificity (TaKaRa).

PCR amplification was performed with the following cycling parameters: initial denaturation at 98°C for 2 min, followed by denaturation at 98°C for 10 s, annealing at 58°C for 15 s, extension at 68°C for 40 s, for a total of 35 cycles. The resulting amplicons were purified using the Fast Gene Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan) and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) and 3,130 Genetic Analyzer (Applied Biosystems, Tokyo, Japan) according to the manufacturer's instructions. All ITS sequences were assigned to operational taxonomic units (OTUs) defined by 97% sequence similarity. All ITS sequences were analyzed using BLAST searches (Altschul, 1997) against the GenBank sequence database to find the closest matching sequence. The full-length ITS sequences of each OTU were edited using the ATGC v7 sequence assembly software (Genetyx, Tokyo, Japan) and deposited in the DNA Data Bank of Japan under accession numbers LC597346, LC597350, and LC683198–LC683206.

Phylogenetic analysis

OTUs belonging to Tulasnellaceae, Serendipitaceae and Ceratobasidiaceae, which are known OMF, were considered putative mycorrhizal associates and subjected to phylogenetic analysis using ITS sequences. Sequences obtained from *Dendrobium* species in previous studies were included in the analysis (Wang et al., 2011; Shao et al., 2019; Zhang et al., 2020). The phylogenetic analysis was performed using the MEGA 11 software (Nei and Kumar, 2000; Stecher et al., 2020; Tamura et al., 2021). Maximum likelihood (ML) trees were obtained using the GTR + G + I model. Bootstrap (BS) analysis of the ML trees was performed using 1,000 replicates (Felsenstein, 1985). All positions with < 90% site coverage was eliminated, i.e., < 10% of alignment gaps, missing dates, and ambiguous bases were allowed at any position.

Symbiotic culture

Six fungal isolates from *D. officinale* were used (**Table 2**). A fungal colony of each isolate was transferred onto PDA as pre-culture and cultured in the dark at 25 ± 1°C for 7 days. Seeds were obtained from nine mature capsules from five individuals. Seeds from four to five capsules of two or three individuals were mixed and used for symbiotic culture. Prior to each use, seeds were tested using the TTC (2,3,5-triphenyl tetrazolium chloride) method to ensure high viability (> 90%) (Vujanovic et al., 2000). The collected capsules were sterilized using 75% ethanol and dried for 1 week using silica gel desiccant until they had nearly ruptured. Seeds were collected from the capsules and stored at 5°C until use. Seeds were sterilized with 1% sodium hypochlorite solution for 3 min, sown on oatmeal agar medium (OMA; 2.5 g/L oatmeal and 15 g/L agar) and maintained at 25°C for 1 week for contamination checking. After 1 week without contamination, 1 cm × 1 cm discs were cut (5–10 seeds per disc) and transplanted to new OMA media. A total of 20 seeds on two to four discs were placed on each new medium plate. Each treatment consisted of 5–15 replicates, for a total of 100–300 seeds. A 6-mm plug of fungal culture was inoculated onto the OMA medium, and the cultures were placed under a 12 h/12 h light/dark photoperiod at 25 ± 1°C. Petri dishes without fungal inoculum were prepared as a control. After 90 days of culture, the seeds were counted under a stereomicroscope. Germination and seedling growth and development were scored on a scale of 0–5 as described previously (Stewart et al., 2003; **Table 3**). The data were analyzed by one-way ANOVA and Turkey-Kramer test using IBM SPSS (ver. 27 IBM Corp., NY, USA).

To confirm fungal colonization, the protocorms were cleared using 10% KOH solution, washed in 2% HCl, and stained with 0.05% trypan blue in lactoglycerol, as described previously (Phillips and Hayman, 1970), with modifications. Stained protocorms were de-stained in lactoglycerol prior to microscopic observation (Nikon Eclipse 50i, Nikon, Tokyo, Japan).

Results

Molecular identification of mycorrhizal fungi

In total, 60 root samples and 34 isolates collected from 36 individuals from six sites were analyzed (**Table 1**). In total, 96 fungal sequences were obtained from these samples and 79.2% of the sequences were OMF, including 45.8% Tulasnellaceae, 28.1% Serendipitaceae, 3.1% Ceratobasidiaceae, and 2.1% *Fusarium* (**Figure 1**). Two or three different sequences were obtained from each of the six samples using different primer

TABLE 3 Seed germination and protocorm development in *Dendrobium officinale*.

Stage description	
Stage 0	No germination, viable embryo
Stage 1	Enlarged embryo
Stage 2	Continued embryo enlargement, rupture of testa
Stage 3	Appearance of protomeristem
Stage 4	Emergence of first leaf
Stage 5	Growing two leaves or a root

sets. The independent data sets of fungal sequences for root samples and isolates showed that both data sets consisted of Tulasnellaceae, Serendipitaceae and Ceratobasidiaceae (**Supplementary Figure 1**). The OMF sequences were assigned to 10 OTUs including six Tulasnellaceae, two Serendipitaceae, one Ceratobasidiaceae, and one *Fusarium* (**Figure 2**). Two *Fusarium* sequences obtained in this study showed high sequence similarity (99–100%) with *Fusarium oxysporum* according to BLAST analysis. This species formed fungal coils in *D. candidum* root cells (Jiang et al., 2019) and has been sampled from *D. officinale* seedlings (Chen et al., 2021). Therefore, we added these *Fusarium* sequences to the OMF OTUs as FU1.

Mycorrhizal fungi were compared among sites and substrates (**Figure 2**). We collected *D. officinale* samples from six different sites and 11 substrates. The dominant mycorrhizal fungi varied among both sites and substrates, even within the same site. SE1 was the most frequently detected OTU, occurring in 26 samples from four sites and accounting for 27.1% of all detected fungal OTUs (**Figure 1**). The second most frequently detected OTU was TU27, which was found in 15 samples from two sites, accounting for 15.6%. TU22 was detected in 9 samples from two sites (9.4%), and TU10 in 7 samples from three sites (7.3%).

The relative frequencies of Serendipitaceae and Tulasnellaceae differed greatly between lithophytic and

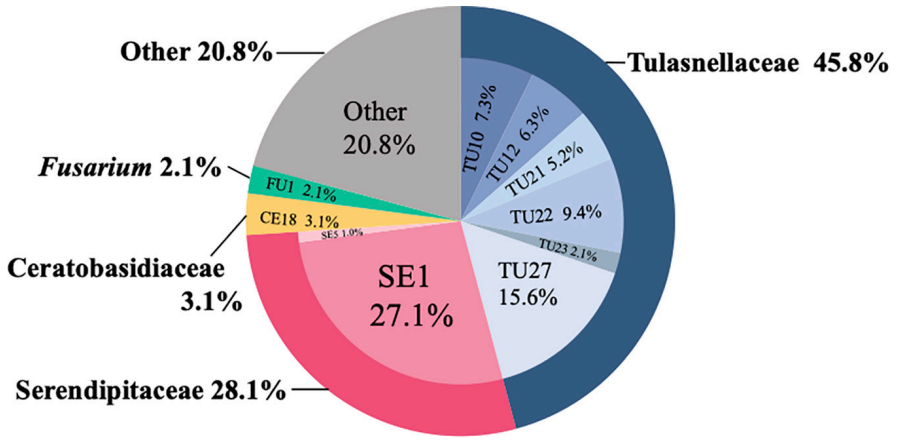

FIGURE 1
Frequency distribution of fungal sequences identified from *Dendrobium officinale* using 96 sequences. Identical sequences obtained from a single sample using different primer pairs were discarded.

Site No.	Habitat	Substrate	Detected fungal OTUs									
			Tulasnellaceae						Serendipitaceae		Ceratobasidiaceae	*Fusarium*
			TU10	TU12	TU21	TU22	TU23	TU27	SE1	SE5	CE18	FU1
S1	L	Cement block wall										
	E	*Aesculus turbinata*										
S2	L	Cement bridge										
	E	*Castanopsis cuspidata*										
	E	*Glochidion obovatum*										
	L	Cement bridge										
S3	L	Rock wall										
S4	L	Cement bridge										
	E	*Athruphyllum neriifolium*										
	L	Cement roof										
	E	*Quercus salicina*										
S5	E	Unknown fallen tree										
S6	E	*Ficus superba*										
				1		2 ~ 4		5 ~ 7				

FIGURE 2
Binary matrix showing the relationship between the sampling sites, substrates, and detected fungal operational taxonomic units (OTUs). The abundance of detected OTUs is indicated as a gradient from white to black. L indicates lithophytic and E indicates epiphytic habitats.

epiphytic individuals (Figure 3). Serendipitaceae accounted for 47.3% of the total in lithophytes (Figure 3A) and only 4.8% in epiphytes (Figure 3B). By contrast, Tulasnellaceae accounted for 43.6% in lithophytes and 95.2% in epiphytes. Among the 10 detected OTUs, four (TU10, TU22, TU23, and TU27) were present in both substrates, whereas four (TU12, SE1, CE18, and FU1) and two (TU21 and SE5) OTUs were unique to lithophytes and epiphytes, respectively (Figure 3C). Serendipitaceae found in lithophytes consisted of only a single OTU, SE1, which was unique to lithophytes and accounted for approximately half of the total frequency (Figure 3A). TU27 was dominant in epiphytes, accounting for 52.4% of the total frequency, whereas it accounted for only 7.3% in lithophytes (Figures 3A,B).

Phylogenetic analysis

Phylogenetic analysis of Serendipitaceae was conducted using two Serendipitaceae OTUs obtained in this study and 33 sequences obtained from the GenBank database (Figure 4). The most dominant mycorrhizal fungus, SE1, formed a monophyletic clade of *Thanatephorus* sp. SSCDO-8 (MH348617: 97.2% sequence similarity) from *D. officinale* (as syn. *D. catenatum* in Zhu et al., 2009), with BS = 99%. SE5 was closely related to Sebacinales sp. from *D. officinale* (MN173026) and Sebacinales sp. SSCDO-6 from *D. officinale* (MH348615), sharing 96.9 and 97.3% ITS sequence similarity, respectively.

The ITS sequences of 6 Tulasnellaceae OTUs obtained in this study and 44 obtained from the GenBank database were used to generate the phylogenetic tree (Figure 5). The second dominant mycorrhizal fungus, TU27, formed a monophyletic clade with four Tulasnellaceae sequences from *D. officinale* (MH348611, MH348612, MH348613, and MH348616), sharing 97.8–98.0% ITS sequence similarity, with BS = 98%. The TU22 sequence was closely related to the three mycorrhizal fungal sequences from *D. officinale* (MN545849, MN545657, and MN545858), sharing 96.7–98.2% similarity. TU12 formed a monophyletic clade with two *Tulasnella* sequences from *D. officinale* (EF393629 and MN544859) with BS = 99% and shared 97.0–98.8% similarity. TU10 was clustered with mycorrhizal fungi from epiphytic orchid, *Ascocentrum himalaicum* (JQ713573), with BS = 97%, and closely related to TU27 (BS = 82%). TU23 was clustered with mycorrhizal fungi isolated from other epiphytic species (LC597355, LC568587, OL374168) with BS = 98%. TU21 was closely related to epiphytic species, *Liparis viridiflora* (KP053821), BS = 98%, and distantly related to the other Tulasnellaceae OTUs.

Phylogenetic analysis of Ceratobasidiaceae was conducted using one Ceratobasidiaceae OTU obtained in

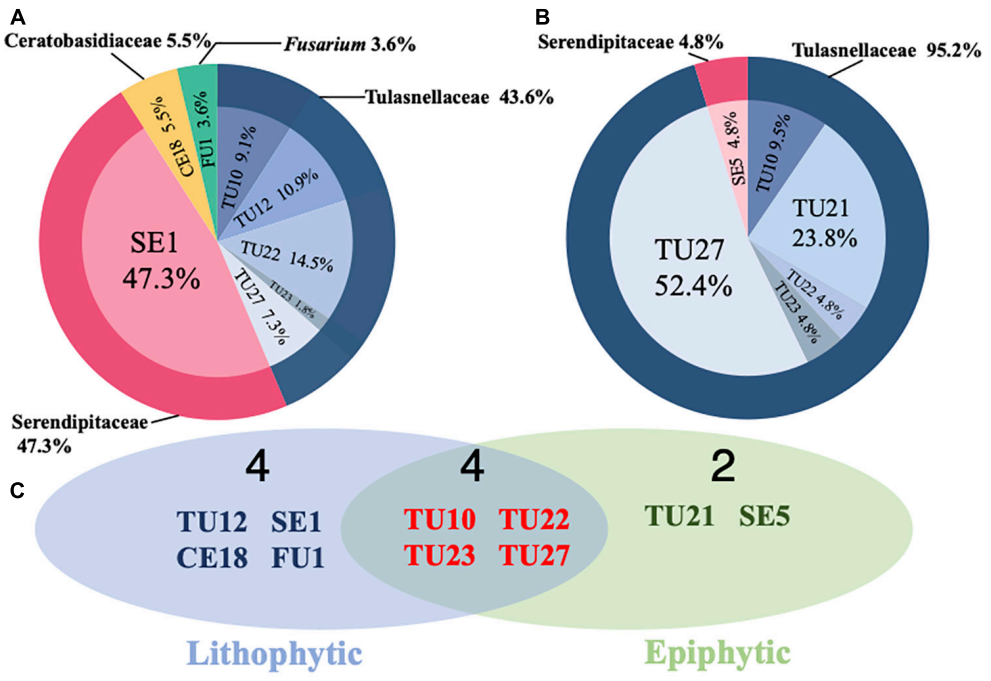

FIGURE 3
Comparison of orchid mycorrhizal fungi (OMF) associating with lithophytic and epiphytic *Dendrobium officinale* individuals. Frequency distribution of OMF sequences detected from **(A)** lithophytic and **(B)** epiphytic individuals. **(C)** Venn diagrams showing the numbers of OMF OTUs.

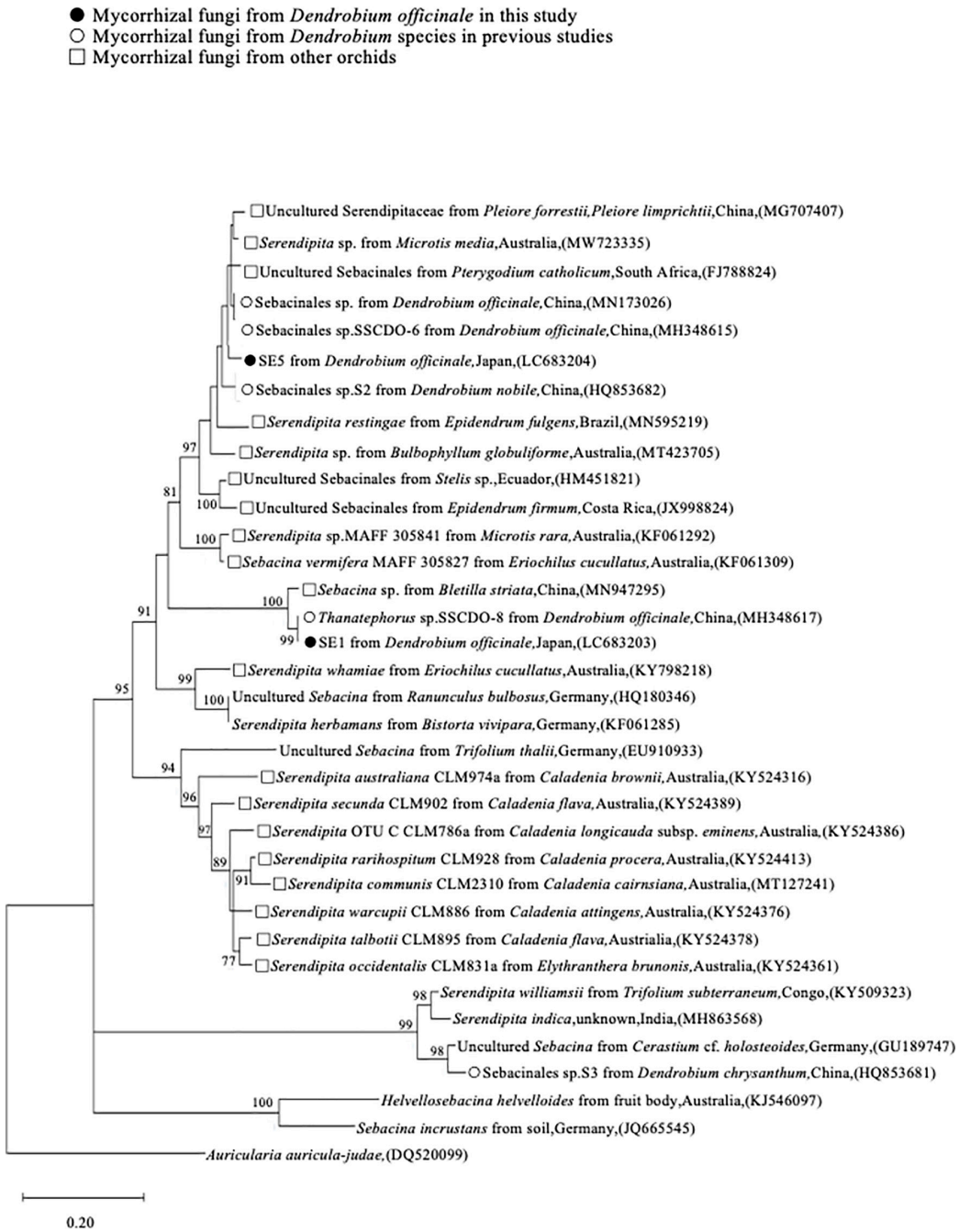

FIGURE 4
Maximum likelihood tree for Serendipitaceae internal transcribed spacer (ITS) sequences, including those of two OTUs from this study. Symbols indicate the origin of each sequence. Only bootstrap values ≥ 70% are shown. The tree is drawn to scale, with branch lengths reflecting the number of substitutions per site. *Auricularia auricula-judae* was used as an outgroup taxon. The 35 assembled sequences were aligned, and the final dataset included 382 bp sequences.

this study and 34 sequences obtained from the GenBank database (**Supplementary Figure 2**). The CE18 formed a monophyletic group with other Genbank sequences divided from mycobionts of epiphytic orchids containing *D. officinale* (JX545227), *Aranda* (AJ318429), *Liparis* (LC278371), terrestrial orchid of *Dactylorhiza* (EF536969) and three sequences from plant pathogens, *Rhizoctonia* sp. AG-G (JF519837, KC825348), *Ceratobasidium* sp. AG-G (DQ102402), sharing 99.5–100% ITS sequence similarity, with BS = 96%.

FIGURE 5
Maximum likelihood tree for Tulasnellaceae ITS sequences, including six OTUs from this study. Symbols indicate the origin of each sequence. Only bootstrap values ≥ 70% are shown. The tree is drawn to scale, with branch lengths reflecting the number of substitutions per site. *Tulasnella alibida* and *Tulasnella hadrolaeliae* were used as outgroup taxa. The 50 assembled sequences were aligned, and the final dataset included 442 bp sequences.

Symbiotic culture

Seeds from *D. officinale* were cultured symbiotically with six OTU isolates including three Tulasnellaceae, two Serendipitaceae, and one Ceratobasidiaceae (**Table 2**). After 3 months of culture, all isolates except for CE18 promoted seed germination to different degrees (**Table 4**). Seeds inoculated with TU22, TU27, SE1, and SE5 developed at stage 5, and TU22 and SE1 showed higher development rates than the other isolates. TU10 also promoted seed germination, with seeds developing at stage 4. Seeds cultured with CE18 became swollen and did not develop past stage 2. All six isolates formed intracellular hyphal coils in protocorm cells (**Supplementary Figure 3**).

Discussion

In this study, 10 OTUs were detected as OMF in *D. officinale* samples collected from six sites; eight were included in Tulasnellaceae and Serendipitaceae, accounting for 73.9% of all detected fungal sequences (**Figures 1**, **2**). This implies that these fungal families are the most dominant fungal partners of *D. officinale*. Most previous studies of *D. officinale* sampled from southern China have also found Tulasnellaceae and/or Serendipitaceae in roots or protocorms germinated *in situ* (Wang et al., 2011; Wu et al., 2012; Shao et al., 2019). These results imply that independent of its distribution range, *D. officinale* has mycorrhizal associations mainly with Tulasnellaceae and Serendipitaceae fungi. Phylogenetic analysis showed that three of the six Tulasnellaceae OTUs and two Serendipitaceae OTUs showed greater than 97% sequence similarity to mycorrhizal fungi associated with *D. officinale* from China (**Figures 4**, **5**). These OTUs include the most frequent OTUs detected in this study, SE1 and TU27 (**Figure 1**). Although *D. officinale* is associated with a wide range of basidiomycetous mycorrhizal partners, its main fungal partners may be widely shared among *D. officinale* populations. In *Dendrobium okinawense*, 11 mature plants from four sites were predominantly associated with a single Tulasnellaceae OTU (Rammitsu et al., 2021). Such high specificity is also found in *Dendrobium fimbriatum*, which was associated with only two OTUs in 15 root samples from two sites (Xing et al., 2013). Mycorrhizal specificity may vary among *Dendrobium* species (Xing et al., 2017), and *D. officinale* appears to have lower specificity than its congeners.

Orchid mycorrhizal communities of *D. officinale* varied among sites in this study (**Figure 2**). Xing et al. (2013) also found that *D. officinale* from two sites had distinct OMF communities in Guangxi Province, China. Such community differences among sites have also been recorded in terrestrial orchids (Jacquemyn et al., 2012; Kohout et al., 2013; Oja et al., 2015). There is some evidence that soil chemical characteristics such as phosphorus, zinc, and organic matter (Kaur et al., 2021) and nitrogen, phosphorus, and water content (Han et al., 2016), impact OMF communities in orchid roots and soils. These differences in substrate chemical and physical characteristics may vary among sites, resulting in corresponding OMF community differences.

Mycorrhizal community composition differed between lithophytic and epiphytic individuals in this study (**Figure 3**). The dominant mycorrhizal fungus among lithophytes was a Serendipitaceae OTU, SE1, whereas that of epiphytes was a Tulasnellaceae OTU, TU27. Distinct OMF communities between lithophytic and epiphytic individuals were also recorded for the orchid *Coelogyne viscosa* (Xing et al., 2015). Among lithophytic and epiphytic individuals of *Coelogyne corymbosa*, Serendipitaceae fungi contributed a relatively large portion of the OTU communities specific to lithophytic orchids (Qin et al., 2020). Yokoya et al. (2021) surveyed 11 growing *Cynorkis* orchid species within lithophytic and terrestrial habitats and found that Serendipitaceae OTUs were frequently found in species inhabiting granite/rock, whereas Tulasnellaceae OTUs were found in both habitat types; they also reported that most Serendipitaceae OTUs were found in the habitat with higher phosphorus and nitrogen content, which may indicate that Serendipitaceae prefers soil conditions with high phosphorus and nitrogen levels. These differences in nutrient

TABLE 4 Effects of fungal isolates on *Dendrobium officinale* seed germination and protocorm development after 3 months of culture.

Treatment	Ratio of seed germination and protocorm development (%)[a]					
	Stage 0	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
Control[b]	13.72 ± 1.67a	10.40 ± 1.42b	75.71 ± 2.40d	0.17 ± 0.17a	0.00 ± 0.00a	0.00 ± 0.00a
TU10	10.19 ± 3.35a	1.54 ± 1.54a	13.11 ± 1.45b	54.76 ± 5.25c	20.40 ± 4.83b	0.00 ± 0.00a
TU22	13.30 ± 1.90a	0.00 ± 0.00a	0.69 ± 0.46a	0.00 ± 0.00a	8.77 ± 8.77ab	77.25 ± 7.93c
TU27	13.14 ± 1.71a	3.47 ± 1.67a	9.92 ± 3.86ab	9.77 ± 2.88ab	16.83 ± 3.66ab	46.87 ± 9.22b
SE1	12.45 ± 2.20a	0.21 ± 0.21a	1.74 ± 0.74ab	3.48 ± 1.10ab	10.18 ± 1.65ab	71.94 ± 3.46bc
SE5	13.20 ± 1.92a	0.99 ± 0.74a	5.94 ± 2.09ab	11.28 ± 3.41b	22.84 ± 3.32b	45.75 ± 6.79b
CE18	10.60 ± 2.21a	27.36 ± 2.52c	62.04 ± 2.63c	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a

[a]Germination percentage (mean ± SE, n = 5–15) within columns marked by different letters are significantly different at $P < 0.05$ (Tukey Kramer).
[b]Seeds without fungal inoculation.

conditions may contribute to OMF community differences between lithophytic and epiphytic individuals in *D. officinale*.

Germination of *D. officinale* seeds was promoted by five of the six OMF used in this study (**Table 4**). Although all six OMF formed coiled fungal hyphae within the protocorm cells according to histological observation (**Supplementary Figure 3**), the ability to promote seed germination varied greatly among OMF (**Table 4**). All OMF, except CE18, exhibited germination-promoting effects, and seedlings with TU22, TU27, SE1, and SE5 were able to reach stage 5. Phylogenetic analysis showed that the sequences of these four OTUs shared ≥ 97% sequence similarity with fungal isolates obtained in previous studies of *D. officinale* (**Figures 4**, **5**). Tulasnellaceae sp. SSCDO-7, which is closely related to TU27 (**Figure 5**), strongly promotes seed germination in *D. officinale* (Shao et al., 2019). Tulasnellaceae sp. TPYD1, TPYD2, and TPYD3, which share 97–98% sequence similarity with TU22, promote the growth of *D. officinale* seedlings produced *in vitro* (Chen et al., 2021). Serendipitaceae isolates SSCDO-8 and SSCDO-6, which are closely related to SE1 and SE5, respectively, also induce *D. officinale* seed germination and seedling growth (Shao et al., 2019). Our molecular analysis showed that SE1, TU27, and TU22 were the most frequent fungal OTUs in adult individuals (**Figure 1**), and these fungi promoted seed germination and protocorm development (**Table 4**). These results suggest that the main fungal partners at the adult stage can induce seed germination and support seedling development in *D. officinale*.

Seedlings with TU10 developed at stage 4 after 3 months of culture (**Table 4**) and continued growth, reaching stage 5 after 6 months (data not shown). This fungus induced seed germination, but with slower seedling growth than other effective fungal isolates. By contrast, seedlings with CE18 reached stage 2 after 2 months and showed no further growth, despite our detection of coiled fungal hyphae in protocorm cells (**Supplementary Figure 3**). Hence, this fungal strain appears not to contribute to seed germination in *D. officinale*. Ceratobasidiaceae fungi are considered important partners of other orchid genera such as *Goodyera* (Shefferson et al., 2010), *Tolumnia* (Otero et al., 2004), and *Pterostylis* (Bougoure et al., 2005; Bonnardeaux et al., 2007). Phylogenetic analysis showed that CE18 was closely related to OMF from epiphytic and terrestrial orchids (**Supplementary Figure 2**). However, it has rarely been sampled from *D. officinale* roots. Because all root samples bearing the Ceratobasidiaceae sequence were accompanied by Tulasnellaceae or Serendipitaceae sequences in this study, Ceratobasidiaceae may not a main fungal partner for *D. officinale*.

Conclusion

In conclusion, our results demonstrate that *D. officinale* mainly forms OMF with Tulasnellaceae and Serendipitaceae as its main fungal partners, such as SE1 and TU27. These fungal partners induced *D. officinale* seed germination and seedling development *in vitro*, suggesting that they are its main fungal partners throughout its life cycle. The *in situ* seed baiting technique, which was proposed as an effective and simple technique for obtaining seed germination-enhancing fungi *in situ* (Rasmussen and Whigham, 1993), will contribute to a more comprehensive understanding of the mycorrhizal associations of *D. officinale* throughout its life cycle. Our results show that the OMF community differed between lithophytic and epiphytic individuals, suggesting that mycorrhizal specificity may vary by habitat type. Our findings contribute to understanding of mycorrhizal associations among wild *Dendrobium* species, the conservation of endangered *Dendrobium* species, and the industrial production of medicinal *Dendrobium* species.

Data availability statement

Sequence data have been deposited in DNA Data Bank of Japan (DDBJ) under accession numbers LC597346, LC597350, and LC683198–LC683206.

Author contributions

YO-T and TY involved in the study conception and design. KT contributed to the field survey. LZ and KR performed the sampling, experiments, data collection, and analysis. LZ and YO-T wrote the manuscript. All authors commented on previous versions of the manuscript, read, and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Delimiting species in the taxonomically challenging orchid section *Pseudophrys*: Bayesian analyses of genetic and phenotypic data

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Accurate species delimitation is critical for biodiversity conservation. Integrative taxonomy has been advocated for a long time, yet tools allowing true integration of genetic and phenotypic data have been developed quite recently and applied to few models, especially in plants. In this study, we investigated species boundaries within a group of twelve *Pseudophrys* taxa from France by analyzing genetic, morphometric and chemical (*i.e.*, floral scents) data in a Bayesian framework using the program integrated Bayesian Phylogenetics and Phylogeography (iBPP). We found that these twelve taxa were merged into four species when only genetic data were used, while most formally described species were recognized as such when only phenotypic (either morphometric or chemical) data were used. The result of the iBPP analysis performed on both genetic and phenotypic data supports the proposal to merge *Ophrys bilunulata* and *O. marmorata* on the one hand, and *O. funerea* and *O. zonata* on the other hand. Our results show that phenotypic data are particularly informative in the section *Pseudophrys* and that their integration in a model-based method significantly improves the accuracy of species delimitation. We are convinced that the integrative taxonomic approach proposed in this study holds great promise to conduct taxonomic revisions in other orchid groups.

KEYWORDS

integrative taxonomy, species delimitation, iBPP, floral scents, orchids

Introduction

Accurately delimiting species is of critical importance for many fields of research in biology, including conservation biology. Species are commonly defined as independently evolving linages that can be delimited using various criteria (Hey, 2006; De Queiroz, 2007). As any single line of evidence may fail at detecting species

boundaries (Knowles and Carstens, 2007), many authors have advocated the use of an integrative approach combining several lines of evidence, both genetic and phenotypic (Dayrat, 2005; Will et al., 2005; Padial et al., 2010; Pires and Marinoni, 2010). However, until recently, genetic and phenotypic data were almost always integrated in a purely qualitative way, as no quantitative methods were available for processing simultaneously both data types (Schlick-Steiner et al., 2010; Yeates et al., 2011). Fortunately, model-based species delimitation methods, which were originally developed for DNA sequences (Fujita et al., 2012; Naciri and Linder, 2015), were later extended to integrate quantitative traits (Guillot et al., 2012; Solís-Lemus et al., 2015), thereby improving objectivity and repeatability of integrative species delimitation. Such methods have been applied to various animal (Huang and Knowles, 2016; Pyron et al., 2016; Olave et al., 2017; Núñez et al., 2022) and plant (Yang et al., 2019; Zhang et al., 2020) clades and have proven useful in several cases. Because model-based species delimitation methods may cause oversplitting when solely based on genetic data (Sukumaran and Knowles, 2017; Mason et al., 2020), combining the latter with phenotypic data may provide more conservative estimates of species numbers (*e.g.,* Pyron et al., 2016). Conversely, in recently radiating clades, in which species often lack clear genetic differentiation, integrating morphological or ecological data may increase the power to detect species boundaries (*e.g.,* Edwards and Knowles, 2014; Solís-Lemus et al., 2015).

Hyperdiverse clades deserve particular conservation attention but may be taxonomically challenging. This is, for example, the case of the Orchidaceae family, which comprises more than 30,000 named species [Plants of the World Online [POWO], 2022], including some of the most threatened species in the world (Fay, 2018), but in which species boundaries are sometimes blurred (Barrett and Freudenstein, 2011; Pessoa et al., 2012). Within this family, the Mediterranean genus *Ophrys* L. is of particular interest, due to its high level of ecological specialization and endemism rate, but it is also considered as a textbook example of taxonomic confusion (Bertrand et al., 2021; Cuypers et al., 2022), which may affect its conservation (Agapow et al., 2004; Pillon and Chase, 2007; Vereecken et al., 2010; Schatz et al., 2014). Some of this confusion arises from conflicting views on which operational criteria should be used to delimit species in this genus. Specifically, some authors support that taxa should have achieved reciprocal monophyly (Devey et al., 2008; Bateman et al., 2011) to be considered as "good" species, while others argue that interactions between *Ophrys* and pollinators are more informative than neutral markers due to their key role in speciation (Schiestl and Ayasse, 2002; Ayasse et al., 2011; Vereecken et al., 2011; Baguette et al., 2020). Indeed, *Ophrys* species attract one or a few pollinator species (Joffard et al., 2019; Schatz et al., 2020) using sex pheromones-mimicking floral scents (Schiestl et al., 1999; Ayasse et al., 2003). In these species, changes in floral scents may cause pollinator shifts, which may in turn mediate reproductive isolation between conspecific populations and drive speciation (Sedeek et al., 2014). Distinct views on which criteria should be used to delimit species has led to the recognition of dozens (Devey et al., 2008) *versus* hundreds (Paulus, 2006) of *Ophrys* species. In addition, even authors who favor the same criteria sometimes disagree on where along the speciation continuum independently evolving lineages should be recognized as species, *i.e.,* "splitters" (*e.g.,* Devillers and Devillers-Terschuren, 1994; Delforge, 2016) *versus* "lumpers" (*e.g.,* Pedersen and Faurholdt, 2007; Kühn et al., 2020). In this context, model-based species delimitation methods integrating genetic and phenotypic data could be particularly helpful.

In this study, we aim at delimiting species through the integration of molecular markers, morphometric characters and floral scents in a group of twelve *Pseudophrys* taxa. We compare species boundaries based on genetic and phenotypic data alone or in combination and we discuss the potential of integrative taxonomy in solving long-standing debates about *Ophrys* taxonomy.

Materials and methods

Studied species and populations

The monophyletic section *Pseudophrys* Godfery comprises twelve groups, each of them including one to twelve taxa (Delforge, 2016). Here, we focused on the twelve *Pseudophrys* taxa that are described in France (**Table 1** and **Figure 1**; Bournérias and Prat, 2005). Among them, eight belong to the *O. fusca* group (namely *O. bilunulata*, *O. delforgei* subsp. "*O. forestieri*" sensu neotypus 1999, *O. funerea*, *O. lupercalis*, *O. marmorata*, *O. peraiolae*, *O. sulcata,* and *O. zonata*), one to the *O. iricolor* group (*O. eleonorae*), two to the *O. lutea* group (namely *O. corsica* and *O. lutea*) and one to the *O. omegaifera* group (*O. vasconica*). These twelve taxa differ in their geographical distribution, some of them being widely distributed (*e.g., O. bilunulata*, *O. lupercalis,* and *O. lutea*), while others have restricted distribution areas, *e.g.,* in South-eastern France (*O. deforgei*), South-western France and Northern Spain (*O. vasconica*) or Corsica and Sardinia (*O. corsica*, *O. eleonorae*, *O. funerea*, *O. marmorata*, *O. peraiolae,* and *O. zonata*). By contrast, these twelve taxa do not strongly differ in their flowering phenology or habitats: except for *O. sulcata* and *O. vasconica*, they all flower in early spring and grow in open, dry habitats typical of the Mediterranean region (Bournérias and Prat, 2005). Among them, *O. lupercalis, O. lutea, O. sulcata,* and *O. vasconica* are regionally protected in France (Bournérias and Prat, 2005) and several of them are currently considered as threatened at the national or regional level, such as *O. eleonorae* (considered as endangered at the national level and as critically endangered in the Corsican region) and

TABLE 1 Number of populations and individuals sampled for molecular, morphometric, and chemical data.

Taxon	Population	Molecular data	Morphometric data	Chemical data
Ophrys bilunulata Risso (1844)	Gruissan, 11, France	3 (1)	15	10
	Clapier, 34, France	3 (1)	10	5
	La Gaude, 06, France	2 (1)	–	–
Ophrys corsica Soleirol ex Foelsche and Foelsche (2002)	Bonifacio, 2A, France*	1	25	15
Ophrys delforgei Devillers-Terschuren and Devillers (2006)	Martigues, 13, France*	2	25	20
Ophrys eleonorae Paulus and Gack (2004)	Antisanti, 2B, France	1	–	2
Ophrys funerea Viviani (1824)	Palasca, 2B, France	3 (2)	20	9
	Corte, 2B, France	3 (2)	15	9
	Laconi, Sardinia, Italy	2	–	–
Ophrys lupercalis Devillers and Devillers-Terschuren (1994)	Armissan, 11, France*	2	0	0
	Saint Bauzille de Montmel, 34, France	2 (1)	20	15
	Saint-Florent, 2B, France	–	5	5
Ophrys lutea Cavanilles (1793)	Montferrier sur Lez, 34, France	3 (1)	10	10
	Montarnaud, 34, France	3 (1)	20	10
	Cassis, 13, France	1	–	–
	Maala, Kabylia, Algeria	1	–	–
	Benicolet, Valencian community, Spain	1	–	–
	Sempere, Valencian community, Spain	1	–	–
Ophrys marmorata Foelsche and Foelsche (1998)	Bonifacio, 2A, France*	5 (3)	20	8
Ophrys peraiolae Foelsche et al. (2000)	Palasca, 2B, France*	3	15	8
Ophrys sulcata Devillers and Devillers-Terschuren (1994)	Lapanouse, 12, France	3 (2)	25	10
	Oléron, 17, France*	2	–	–
	Vence, 06, France	1	–	–
Ophrys vasconica Delforge (1991)	Belpech, 11, France	1	20	15
Ophrys zonata Devillers and Devillers-Terschuren (1994)	Saint-Florent, 2B, France	3 (1)	25	15

*: Populations located at the locus classicus. (): Number of new*ly-published* sequences.

O. marmorata (considered as vulnerable in the Corsican region) (IUCN et al., 2010; Delage and Hugot, 2015).

Four hundred ninety individuals belonging to one to six populations per taxon were selected and sampled for molecular, morphometric, or chemical analysis between 2013 and 2016 (**Table 1**). Within populations, molecular, morphometric, and chemical data were not collected on the same individuals as the iBPP program (see below) requires independence of genetic and phenotypic data. Molecular data were collected in one to six populations per taxon, distributed over most of their geographic range, in up to five individuals per population. Morphometric and chemical data were collected in one or two of these populations only, but on up to 25 individuals per population. One population was sampled at the *locus classicus* (*i.e.,* site where the species was described for the first time) for six of these twelve taxa.

Genetic data collection and analysis

One leaf of one to five individual(s) per population were collected between 2014 and 2017 and dried in silica gel for a few days. DNA extraction was performed with a Plant Minikit (®Quiagen). Three genes were amplified and sequenced in 52 individuals: the internal transcribed spacers (ITS) 1 and 2, the first intron of the beta-galactosidase-like (BGP) gene and the first intron of the LEAFY/FLORICULA (LFY) gene. For 36 individuals, sequences have been published in Joffard et al. (2020), while for 16 individuals, sequences are published for the first time in this study (**Supplementary Table 1**).

Polymerase chain reaction (PCR) and sequencing were carried out as described in Joffard et al. (2020). Sequences were edited using CodonCode Aligner v.4.2.7 (CodonCode Corporation). Uncertainties and alleles from heterozygous individuals were merged into consensus sequences using International Union for Pure and Applied Chemistry (IUPAC) coding. Consensus sequences were aligned using the Muscle algorithm (Edgar, 2004) as implemented in SeaView v.4.4.2 (Gouy et al., 2010) prior to concatenation.

A phylogenetic analysis was performed on the concatenated alignment using MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003). *Ophrys cinerophila* from Samos (Greece) was used as outgroup based on Joffard et al. (2020). The best partitioning scheme and the best model for each partition was chosen

FIGURE 1
Photographs of the 12 French *Pseudophrys* taxa sampled for molecular, morphometric, and chemical data. From left to right and top to bottom: *Ophrys bilunulata, O. delforgei, O. funerea, O. lupercalis, O. marmorata, O. peraiolae, O. sulcata* and *O. zonata* **(*O. fusca* group)**, *O. eleonorae* **(*O. iricolor* group)**, *O. corsica, O. lutea* **(*O. lutea* group)**, and *O. vasconica* **(*O. omegaifera* group)**. © N. Joffard and B. Schatz.

using the Bayesian Information Criterion (BIC) as estimated by PartitionFinder v.1.1.1 (Lanfear et al., 2012). Bayesian analysis was conducted with two separate runs of four Markov chain Monte Carlo (MCMC) chains for 10 million generations with tree sampling every 1,000 generations. 25% of the sampled trees were discarded as burn-in, and the 75% best scoring trees were used to calculate the consensus tree.

A DNA barcoding analysis was performed on the concatenated alignment using the Automatic Barcode Gap Detection (ABGD) website (Puillandre et al., 2012). ABGD is a tool designed to infer species hypotheses based on automatized identification of barcode gaps between intra- and interspecific pairwise distances. It aims at revealing a significant barcoding gap in the distribution of pairwise genetic distances, reflecting a discontinuity between intra- and interspecific distances among individuals. ABGD partitions individuals into groups in a recursive manner until no further splits are possible, while integrating priors on maximum and minimum intraspecific differentiation and barcode gap width. In this study, pairwise distances were computed as K2P-corrected distances. We left the default values of 10 steps from Pmin = 0.001 to Pmax = 0.1 for number of steps and intraspecific differentiation, and the default value of 1.5 for barcode gap width.

Phenotypic data collection and analysis

For morphometric data, fifteen to thirty-five individuals per taxon were sampled in 2015 and 2016 (Table 1). *Ophrys eleonorae* was not sampled for morphometric data as no flowering individuals were found in 2015 nor in 2016. However,

this species is known to be morphologically distinct from the eleven other taxa in that it has a particularly long (~15 to 25 mm) and wide (~10 to 20 mm) labellum (Bournérias and Prat, 2005). In each individual, twelve morphometric characters were measured to the nearest 0.01 mm in the field using a digital caliper. Four of these characters concerned the labellum, whose size and shape are important because they must match those of the pollinator, but the length and/or width of the stigmatic cavity, lateral petals, and sepals were also measured (**Supplementary Figure 1**).

For chemical data, eight to twenty individuals per taxon were sampled for floral scents in 2014 and 2015 (**Table 1**) using solid phase microextraction (SPME) (except in the case of *O. eleonorae* in which only two individuals were sampled) as described in Joffard et al. (2016). Floral scents were then analyzed by GC-MS using a Shimadzu QP2010 Plus gas chromatograph-mass spectrometer with an OPTIMA® 5-MS capillary column (30 m × 0.25 mm × 0.25 µm, Macherey-Nagel, Düren, Germany) and helium as carrier gas with the method described in Joffard et al. (2016). Retention times of a series of *n*-alkanes (Qualitative retention time mix, ASTM, Sigma Aldrich®) were used to convert retention times into retention index. Compounds were identified based on retention index and mass spectra which were compared to those recorded in databases (NIST, 2007, Wiley Registry 9th) and in the literature (Adams, 2007) and, for some of them, to retention index and mass spectra of analytical standards. Peak areas were measured with the software GCMSsolution (4.11) (Shimazu®).

Two partial least square discriminant analyses (PLS-DA) were performed, one for morphometric characters and one for floral scents. PLS-DA was chosen over Principal Component Analysis (PCA) because it is suitable for data that are non-independent (due to allometry in the case of morphometric characters and shared biosynthetic pathways in the case of floral scents). Because variances were non-homogenous among compounds, floral scents data were centred log-ratio-transformed prior to analysis. Statistical analyses were performed in R version 3.1.2 (R Development Core Team, 2008).

IBPP species delimitation

A joint Bayesian inference based on genetic and phenotypic data was used to delimit species using the program iBPP v.2.1.3 (Solís-Lemus et al., 2015). This program is an extension of the multispecies coalescent model-based program BPP (Rannala and Yang, 2003; Yang, 2015) which includes models of evolution for phenotypic data under a Brownian Motion (BM) process. Because the program assumes independence of phenotypic data, scores on the first two (for morphometric characters) and five (for floral scents) components resulting from two preliminary PCA were included in the analysis. Note that given the role of floral scents in pollinator attraction, these scents may not have evolved according to the assumptions of a BM process, but the results of the program iBPP have been shown to be robust to such a violation (Solís-Lemus et al., 2015). The program begins with a strictly bifurcating guide tree, which in our case was constructed with the software MrBayes (see above), and collapses internal nodes sequentially. We used a prior gamma distribution G (2, 2,000) for τ (branch lengths) and θ (product of Ne the population size and μ the mutation rate) for genetic data and left the default values of 0 for σ^2 (variance) and λ (within/between species ratio) for phenotypic data (non-informative priors). A reversible MCMC analysis was ran over 1,00,000 generations, sampled every ten generations, with 1,000 generations (10%) discarded as burn-in. Seven analyses were performed: (i) with genetic data only, (ii) with morphometric data only, (iii) with chemical data only, (iv) with both genetic and morphometric data, (v) with both genetic and chemical data, (vi) with both morphometric and chemical data, and (vii) with the entire dataset. Because phylogenetic relationships between the taxa *O. bilunulata*, *O. delforgei,* and *O. marmorata*, as well as between the taxa *O. funerea*, *O. sulcata,* and *O. zonata* could not be resolved, several alternative topologies were tested for the guide tree and the topology that gave the most conservative species delimitation model for these two triplets was retained. The robustness of the results was tested by analyzing the data with both the fine tune settings of zero and one (Yang and Rannala, 2010), and by repeating each analysis five times.

Results

Genetic data

ITS, BGP, and LFY sequences were obtained for 52, 49, and 52 individuals, respectively (153 sequences, including 45 that are newly published). Sequences were obtained for at least three individuals per taxon, except for *O. corsica*, *O. eleonorae,* and *O. vasconica* (sequences obtained for one individual only). ITS, BGP, and LFY sequences contained 73, 562, and 603 parsimony-informative sites on 809, 948, and 2,210 sites, respectively. The phylogenetic tree (**Figure 2**) was congruent with the one described in Joffard et al. (2020), with two well-supported clades, one comprising the taxa *O. lutea*, *O. corsica*, *O. lupercalis*, *O. peraiolae*, *O. delforgei*, *O. bilunulata,* and *O. marmorata* and one comprising the taxa *O. eleonorae*, *O. vasconica*, *O. sulcata*, *O. funerea,* and *O. zonata*. On the nine taxa for which several individuals were sampled for molecular analyses, only two - namely *O. lutea* and *O. peraiolae* - were found to be monophyletic with a posterior probability of 0.98 and 1, respectively. The AGBD method detected three species only: it recognized *O. eleonorae* as a species but merged *O. lutea*, *O. corsica*, *O. lupercalis*, *O. peraiolae*, *O. delforgei*, *O. bilunulata,* and *O. marmorata* on the one hand, and *O. vasconica*, *O. sulcata*,

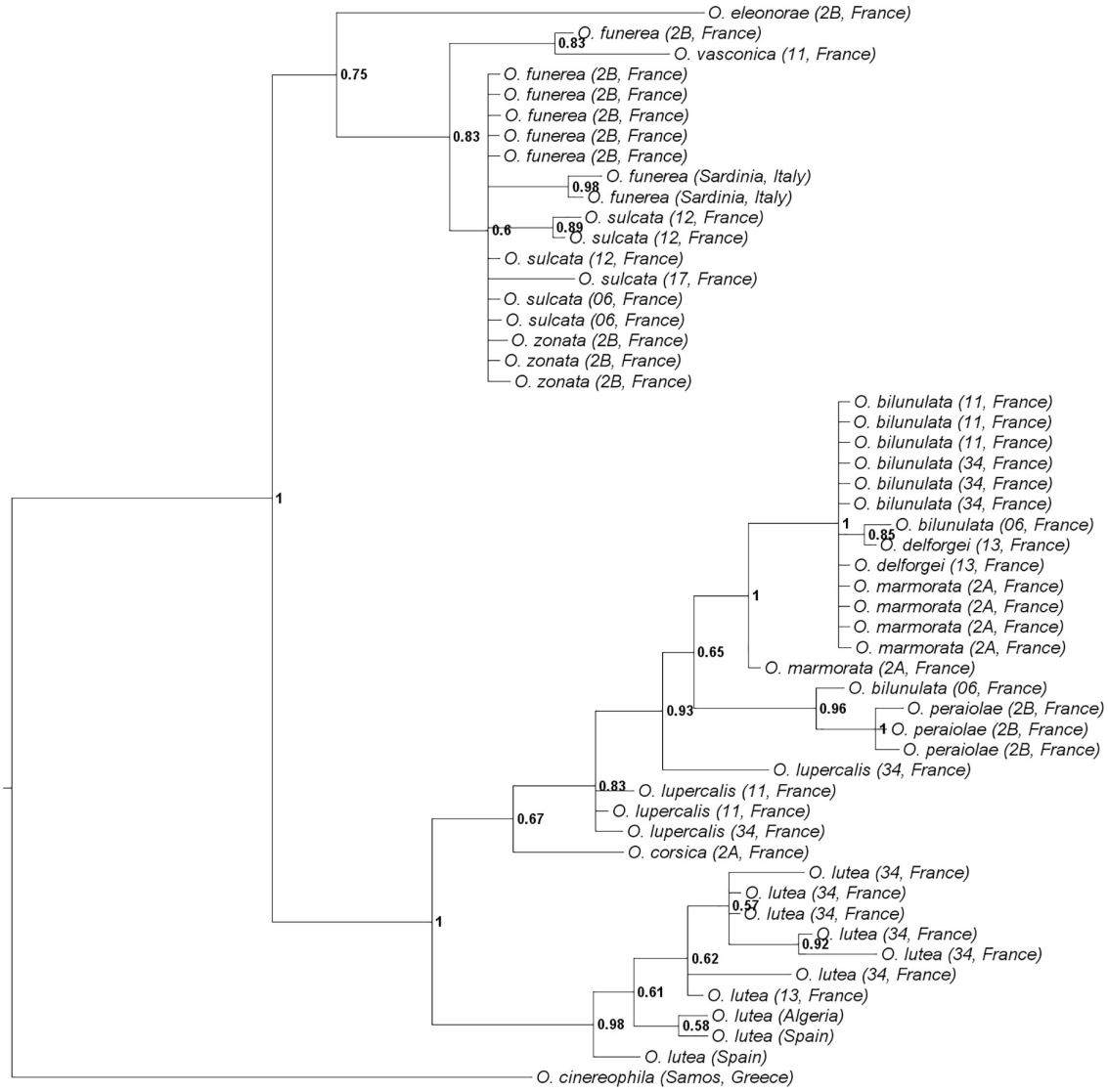

FIGURE 2
Phylogenetic relationships between the twelve French *Pseudophrys* taxa represented by the 50-majority rule consensus tree from the MrBayes analysis. Posterior probabilities are indicated at each node.

O. funerea, and *O. zonata* on the other hand. Mean K2P-corrected distances between individuals were of 2.38 × 10^{-3} substitutions per site within and 7.09 × 10^{-3} substitutions per site between these species. The barcoding gap was located between 3.00 × 10^{-3} and 4.00 × 10^{-3} substitutions per site (**Supplementary Figure 2**).

Morphometric data

Labellum length ranged from 6.11 to 14.35 mm, with a mean of 9.11 (±1.45) mm, and labellum width from 5.88 to 14.23 mm, with a mean of 8.98 (±1.51) mm. *Ophrys lupercalis* and *O. vasconica* were characterized by large sepals, petals, and labella compared to other species, while *O. sulcata*, *O. funerea,* and *O. zonata* were characterized by long petals and sepals but a relatively small labellum, with a high length/width ratio. By contrast, *O. bilunulata*, *O. marmorata,* and *O. peraiolae* were characterized by larger labella with a lower length/width ratio. Finally, the yellow-flowered *O. corsica* and *O. lutea* were characterized by short sepals and petals and a short but wide labellum (**Figure 3** and **Supplementary Table 2**).

Chemical data

Over one hundred VOCs were detected in the blends of the twelve studied taxa, mostly alkanes (19), alkenes and alkadienes

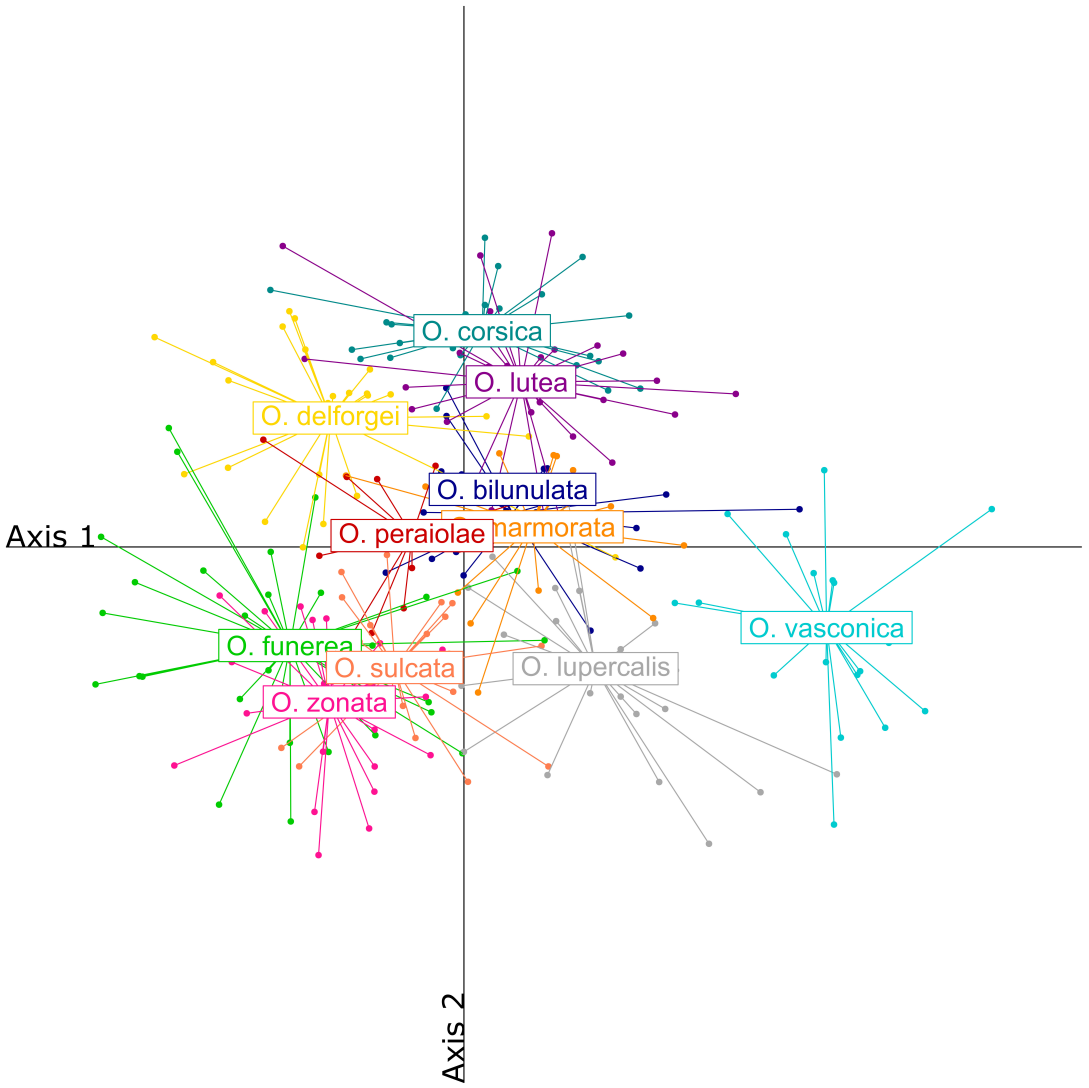

FIGURE 3
Partial least squares-discriminant analysis (PLS-DA) of morphometric characters measured on the twelve French *Pseudophrys* taxa.

(29), aldehydes (23), acids (13) and fatty acid esters (24) (**Supplementary Table 3**). Blends were dominated by alkenes and alkadienes (58.6%) as well as alkanes (28.2%), but aldehydes and fatty acid esters both accounted for more than 5% of the blends. The blends of the *O. fusca*, *O. iricolor*, *O. omegaifera,* and *O. lutea* groups were well differentiated, both qualitatively and quantitatively (**Figure 4**). More precisely, taxa from the *O. fusca* group generally did not produce any fatty acid esters, while taxa from the *O. iricolor*, *O. omegaifera,* and *O. lutea* groups produced significant amounts of nonyl, decyl, and octyl esters, respectively. Within the *O. fusca* group, some species also had well-differentiated blends, although this differentiation was often quantitative rather than qualitative. By contrast, several taxa, such as the *O. funerea*/*O. zonata* pair, produced very similar floral scents.

IBPP species delimitation

Results of iBPP analyses (best species delimitation models, with their respective species numbers and posterior probabilities) are summarized **Figure 5** and detailed **Supplementary Table 4**.

Whatever the type of data included in the analysis, the posterior probability of the best model never exceeded 70%, showing relatively weak support for this model compared to the next best ones. When only genetic data were considered, the three best models (*i.e.,* those for which the sum of posterior probabilities exceeded 80%) all recognized *O. lutea*, *O. corsica*, *O. lupercalis*, *O. peraiolae*, *O. deforgei*, *O. bilunulata,* and *O. marmorata* as a single species. They also all considered *O. eleonorae* as a genuine species and merged *O. funerea*

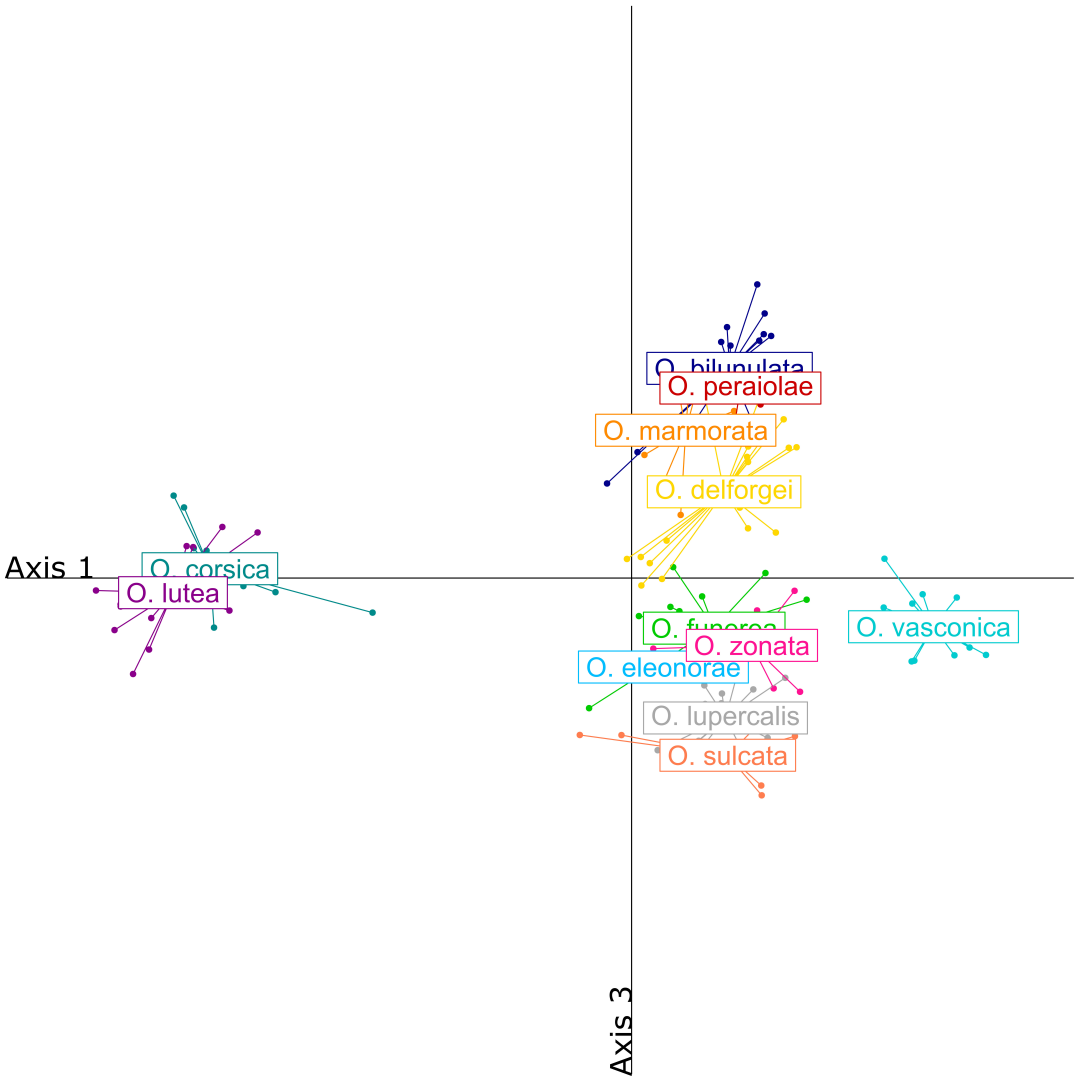

FIGURE 4
Partial least squares-discriminant analysis (PLS-DA) of floral scents detected in the blends of the 12 French *Pseudophrys* taxa. Individuals were represented along axes 1 and 3 to better visualize variation within the *O. fusca* group.

and *O. zonata*. The best model (PP = 61.86%) was a four-species model recognizing *O. vasconica* as a species but merging *O. sulcata* with the *O. funerea*/*O. zonata* pair. By contrast, when only morphometric data were considered, the two best models recognized most taxa as genuine species: the first one (PP = 67.10%) merged *O. bilunulata* and *O. marmorata*, while the second one (PP = 21.98%) delimited twelve species. The same results were obtained when molecular data were included in the analysis, but the posterior probabilities of these two best models decreased, while that of a ten-species model merging both *O. marmorata* with *O. bilunulata* and *O. zonata* with *O. funerea* increased (**Supplementary Table 4**). Likewise, when only chemical data were considered, the two best models were the ten-species model merging both pairs, and an eleven-species model merging *O. zonata* with *O. funerea* only, with comparable posterior probabilities (PP = 47.63 and 44.84%, respectively). Including molecular data in the analysis increased support for the first model (PP = 53.59%) compared to the second one (PP = 39.50%). When only phenotypic (morphometric + chemical) data were considered, the three best models either suggested to merge both the *O. bilunulata*/*O. marmorata* and *O. funerea*/*O. zonata* pairs (PP = 44.83%) or one only (PP = 29.04% for the model merging *O. marmorata* with *O. bilunulata* and 16.02% for the one merging *O. zonata* with *O. funerea*). Finally, the same results were obtained when genetic and phenotypic data were combined, with only slight differences in the posterior probability attributed to each of these three best models compared to the previous analysis (PP = 49.88, 27.63, and 14.19%, respectively).

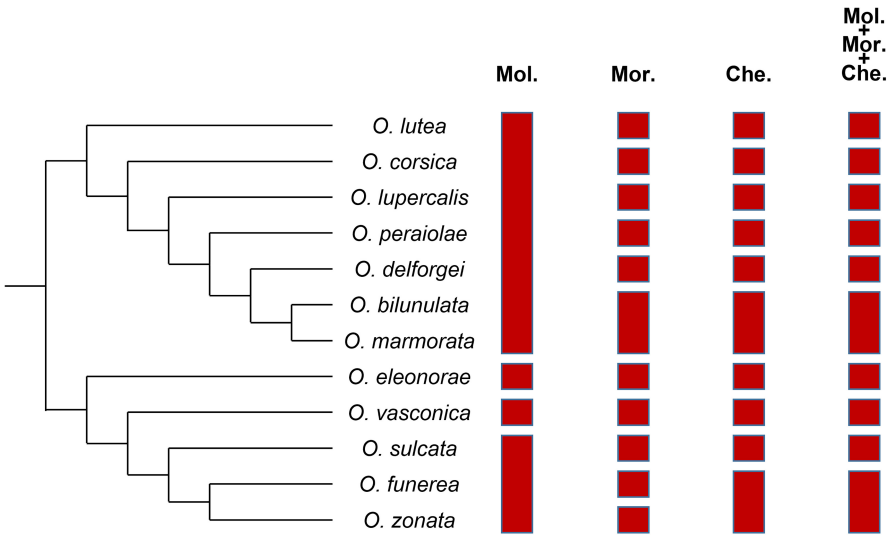

FIGURE 5
Results from the iBPP analysis based on molecular (Mol.), morphometric (Mor.), and chemical (Che.) data analyzed alone or in combination.

These results were robust to the algorithm that was used to collapse internal nodes, and repetitions of each analysis gave similar results. By contrast, these results varied depending on the topology of the guide tree. More specifically, when *O. bilunulata* and *O. marmorata* on the one hand, and *O. funerea* and *O. zonata* on the other hand were not considered as sister-species in the guide tree, the best model was the twelve-species model, because these two taxa were distinct from *O. delforgei* and *O. sulcata*, respectively.

Discussion

Our study aimed at comparing species boundaries drawn from molecular, morphometric, and chemical data alone or in combination in a group of twelve *Pseudophrys* taxa. Our results showed that including phenotypic data in the analysis helped being more accurate when delimiting species in this group. Based on this integrative taxonomic approach, eight formally described species were recognized as such, while the best model suggested merging two pairs of taxa into one species each.

Integration of genetic and phenotypic data in the section *Pseudophrys*

Our results showed that genetic differentiation between the twelve studied taxa was often limited and that species delimitations drawn from genetic data only (using either ABGD or iBPP) were thus very conservative, with only a few taxa recognized as genuine species. Such a limited genetic differentiation could result from incomplete lineage sorting or hybridization (Soliva et al., 2001; Soliva and Widmer, 2003), that are both likely given the recent diversification of the section *Pseudophrys* (Breitkopf et al., 2014; Baguette et al., 2020) and the weakness of post-zygotic barriers between sympatric species (Cortis et al., 2009). Although the markers used in this study were selected because of their high resolution at the scale of the genus, they may not be informative enough to discriminate between such closely related taxa. Just like other non-model organisms, the *Ophrys* genus will likely benefit from the democratization of high-throughput sequencing technics allowing to develop more resolutive markers (*e.g.,* Bateman et al., 2018).

By contrast, phenotypic differentiation between the twelve studied taxa was often significant, perhaps because morphometric characters and floral scents are selected by pollinators and may thus evolve faster and be less affected by hybridization than neutral markers (Sedeek et al., 2014). Indeed, the size and shape of the labellum are likely to be selected to match those of the pollinator's body (Triponez et al., 2013), and floral scents to match sex pheromones of female insects (Schiestl et al., 1999; Ayasse et al., 2003). Interestingly, our results show that morphometric characters are as informative as floral scents to discriminate between *Pseudophrys* species. Both are classically used as criteria to delimit *Ophrys* species (Bernardos et al., 2005; Mant et al., 2005), but in the past decades, much more emphasis has been put on chemical signals (Schiestl et al., 1999; Ayasse et al., 2003; Stökl et al., 2005; Véla et al., 2007). However, our results suggest that using morphometric characters for taxonomic purposes is relevant in the section *Pseudophrys* and emphasize the potential role of orchid enthusiasts in providing valuable data for taxonomic research (Véla et al., 2015). As in the case of molecular markers,

more informative morphometric or chemical markers could be developed using more sophisticated techniques, such as geometric morphometrics (Rakosy et al., 2017; Gibert et al., 2022). More importantly, it would be interesting to distinguish between selected (*i.e.,* functionally significant) and neutral phenotypic traits through electrophysiological and/or behavioral studies (Schiestl et al., 1999; Rakosy et al., 2017). The distinction between biologically active and non-active floral scents, in particular, would likely provide further insights into the taxonomy of the section *Pseudophrys* (Stökl et al., 2005, 2009), as shown in the section *Euophrys* (*e.g.,* Mant et al., 2005).

Because of these heterogeneous levels of resolution between molecular, morphometric and chemical data, species boundaries drawn from genetic *versus* phenotypic data were not congruent. Such an incongruence mirrors disagreements between authors favoring phylogenetic distinctness *versus* reproductive isolation through attraction of distinct pollinator species as a criterion to delimit *Ophrys* species (Paulus, 2006; Devey et al., 2008; Bateman et al., 2010). Methods based on genetic data are often judged more reliable than methods based on phenotypic data because they are not subject to investigator bias, nor affected by environmental or maternal conditions (Fujita et al., 2012). However, speciation sometimes leaves no signature at the level of neutral markers, especially when it is recent and when only a few loci mediate reproductive isolation, as it is assumed to be the case in the genus *Ophrys* (Xu and Schlüter, 2015). In this case, neutral markers-based methods may fail at detecting species boundaries by putting aside the data that are the most informative. Our results also show that integrating several phenotypic traits (in our case, morphometric and chemical) in the analysis may be helpful. For example, in our study, two taxa were slightly distinct morphologically, but strictly similar from a chemical point of view. When only morphometric characters were analysed, these two taxa were recognized as species, whereas when both morphometric characters and floral scents were analysed, they were merged. This shows that integrating new data types – either new molecular markers or new phenotypic traits – may challenge previous taxonomic inferences, species boundaries being hypotheses which should be tested using many data types to increase their robustness (Padial et al., 2010).

Taxonomy and conservation of the section *Pseudophrys*

The section *Pseudophrys* is known to be taxonomically challenging, due to the lack of resolution of classic molecular markers in this section (Schlüter et al., 2007; Devey et al., 2008) and to the striking morphological similarity between its members (Bernardos et al., 2005). Ninety-seven *Pseudophrys* species are described across the Mediterranean region (Delforge, 2016), but the taxonomic rank of many of them has been questioned when confronted to molecular, morphometric or chemical evidence (*e.g., O. arnoldii*: Bernardos et al., 2005; *O. vallesiana*: Gögler et al., 2016). Our analysis supports most *Pseudophrys* species that are described in France, with two remarkable exceptions: on the one hand, the first and second best models suggested to merge *O. marmorata* with the previously described species *O. bilunulata*, and on the other hand, the first and third best models suggested to merge *O. zonata* with the previously described species *O. funerea*. The similarity within these two pairs of taxa has been emphasized before (Bournérias and Prat, 2005; Tison and de Foucault, 2014), *O. marmorata* being sometimes called "*O. bilunulata* from Corsica" (Bournérias and Prat, 2005). Tison and de Foucault (2014) also suggested merging *O. delforgei* with the *O. bilunulata*/*O. marmorata* pair, and *O. sulcata* with the *O. funerea*/*O. zonata* pair, but our analysis does not support these proposals, since we found that both *O. delforgei* and *O. sulcata* were morphologically and chemically distinct from their closest relatives. The continental *O. bilunulata* and the Corsican *O. marmorata* were found to be genetically and phenotypically similar, whereas the Cyrno-Sardinian *O. funerea* and *O. zonata* were found to be slightly distinct morphologically but similar both genetically and chemically. Interestingly, recent records suggest that both *O. bilunulata* and *O. marmorata* are pollinated by *Andrena flavipes* (Schatz et al., 2021), which supports our proposal to merge these two taxa. Likewise, both *O. funerea* and *O. zonata* are pollinated by this species (Foelsche et al., 2000; Schatz et al., 2021), suggesting that these two taxa are not reproductively isolated and should be considered as conspecifics. The proposal to merge *O. marmorata* with *O. bilunulata* does not imply that this taxon should not be considered as vulnerable in Corsica anymore; however, it implies that it should not be considered as threatened at the national level. Another important conclusion of our study is the fact that *O. peraiolae* – which is sometimes described as a morph of *O. marmorata* (Delforge, 2005) and sometimes merged with *O. funerea* and *O. zonata* (Delage and Hugot, 2015) – likely corresponds to a genuine species, although it may be of hybrid origin (Tison and de Foucault, 2014). More generally, our analysis supports many species that are not recognized in the latest version of the European Red List (*e.g., O. lupercalis*, *O. bilunulata*, etc.), in which they are all referred to as "*O. fusca* sensu lato" (Rankou, 2011). We hope that this study will prompt the reassessment of their UICN status and the implementation of appropriate conservation actions, especially for species with extremely restricted distribution areas and declining population sizes such as *O. peraiolae*. We encourage the use of the integrative taxonomic approach proposed in this study to other orchid groups in which species boundaries are blurred, as it provides a framework to interpret patterns of genetic and phenotypic divergence among taxa and would speed up taxonomic revisions that are urgently needed for defining conservation priorities.

Data availability statement

The data presented in the study are deposited in the NCBI GenBank repository, and accession numbers are provided in the **Supplementary material**.

Author contributions

NJ, EV, CM, and BS conceived and designed the analysis. NJ, EV, and BS collected the data. NJ, BB, VA, and CM performed the analysis. All authors discussed the results and contributed to the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Shade and drought increase fungal contribution to partially mycoheterotrophic terrestrial orchids *Goodyera pubescens* and *Tipularia discolor*

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Many photosynthetic plants supplement photosynthetic carbon with fungal carbon, but the mechanisms that govern dependence on mycoheterotrophic carbon are poorly understood. We used exclusion shelters to manipulate water and light availability to plants of the terrestrial orchids *Goodyera pubescens* and *Tipularia discolor*. We tracked changes in $\delta^{13}C$ from photosynthesis and $\delta^{15}N$ acquired from soil-derived inorganic nitrogen versus mycoheterotrophy, along with direct measures of photosynthesis in *T. discolor*. We hypothesized that shade would increase dependence on mycoheterotrophy compared to reference plants, while drought would decrease both photosynthesis and the abundance of potential mycorrhizal fungi. Drought and shade enriched ^{13}C and ^{15}N in both *G. pubescens* and *T. discolor*, compared to control plants, indicating increased fungal contribution to orchid tissues. Physiological measurements of *T. discolor* leaves showed that dark respiration, water use efficiency, and relative electron transport rate did not vary significantly, but shaded plants had greater quantum efficiency, suggesting they were light-limited. Light saturated photosynthesis of *T. discolor* leaves was lower in both shaded and drought-treated plants, indicating lower photosynthetic capacity, and likely greater dependence on mycoheterotrophy and corresponding enrichment in ^{13}C and ^{15}N. This study documented changes in orchid dependence on fungal carbon in response to manipulated environmental conditions. Both shade and drought increased the dependence of both orchids on mycoheterotrophically derived carbon and nitrogen.

KEYWORDS

***Goodyera pubescens*, *Tipularia discolor*, mycoheterotrophy, orchid, mycorrhizae, stable isotope**

Introduction

Between 85 and 92% of land plants obtain nutrients and water from the soil through mycorrhizal associations (Wang and Qiu, 2006; Brundrett and Tedersoo, 2018). For most plants, this association is a two-way exchange, with the plant providing carbon to the fungus in exchange for other resources. Some non-photosynthetic plants, termed fully mycoheterotrophic, obtain carbon from their mycorrhizal fungi (Gebauer and Meyer, 2003; Lallemand et al., 2019), while other mycoheterotrophic plants only initially rely on fungal carbon until they produce green leaves (Leake and Cameron, 2010; Těšitel et al., 2018). However, recently many green photosynthetic plants have been shown to supplement photosynthetic carbon with fungal carbon, and are termed partially mycoheterotrophic (Gebauer and Meyer, 2003) or mixotrophic (e.g., Selosse and Roy, 2008; Hynson et al., 2009; Merckx et al., 2010; Selosse and Martos, 2014). The mechanisms that govern the extent to which plants depend on mycoheterotrophically derived carbon are poorly understood but may be important for understanding the evolution of mycorrhizal associations, especially mycoheterotrophy (Selosse and Roy, 2008; Leake and Cameron, 2010; Wang et al., 2021).

All orchids are mycoheterotrophic at the protocorm life history stage and depend entirely on fungi for carbon and other resources that are required for transition to later life history stages. During the later life history stages, the amount of carbon they derive mycoheterotrophically varies (e.g., Leake, 1994; Rasmussen and Rasmussen, 2007). Most orchids photosynthesize at maturity and are not obligate mycoheterotrophs. However, nearly all orchids continue to associate with mycorrhizal fungi, and largely autotrophic orchids can be partially mycoheterotrophic (e.g., Gebauer and Meyer, 2003; Liebel et al., 2010; Yagame et al., 2012; Selosse and Martos, 2014; Hynson, 2016; Schiebold et al., 2018; Schweiger et al., 2018). Studies of albino and variegated variants of green orchids have also been used to demonstrate the importance of resource movement from mycorrhizal fungi to orchids (Selosse et al., 2004; Lallemand et al., 2019; Suetsugu et al., 2019) and to demonstrate a linear relationship between leaf chlorophyll and fungal contributions to plant carbon (Stöckel et al., 2011). Until recently, orchids were solely assumed to be the beneficiaries of a non-mutualistic association, obtaining carbon from fungi but not providing anything in return (Alexander and Hadley, 1985; Smith and Read, 1997). However, recent studies have shown that species within the genus *Goodyera* provide carbon (C) to mycorrhizal fungi under specific laboratory conditions (Cameron et al., 2006, 2008; Hynson et al., 2009). While the circumstances that dictate the direction of C flow are unclear, it has been speculated that stressors that reduce a plant's photosynthetic ability (e.g., limited light and moisture) may prevent autotrophic carbon acquisition to the extent that orchids will increase the level of resources gained through mycoheterotrophy (Gebauer, 2005; McCormick et al., 2006; Hynson et al., 2009; Preiss et al., 2010). Indeed, Preiss et al. (2010) and Schweiger et al. (2019) demonstrated a correlation between the light quantity and the proportion of carbon that orchids derived from fungi.

Almost all herbaceous species in forests are highly or obligately dependent on mycorrhizal fungi (Brundrett and Kendrick, 1988; Whigham, 2004), and the loss of plant-fungal interactions has negative consequences for physiological processes, including nutrient and water uptake (Hale et al., 2011). Plant-fungal interactions can be disrupted, and the quantity and direction of benefits altered. McCormick et al. (2006), for example, found that individuals of *Goodyera pubescens* lost their mycorrhizal fungi during a drought. Surviving plants subsequently associated with the same or different mycorrhizal fungi, but also suffered higher mortality. The presence of mycorrhizal fungi is also important in non-orchids. Bitterlich et al. (2019) found that mycorrhizal fungi supported increased photosynthesis in tomatoes, but only when there was sufficient light and moisture. In contrast, Zhu et al. (2011) and Cabral et al. (2016) found that mycorrhizal plants maintained higher photosynthetic rates and yield in response to heat stress. These findings suggest that stress responses by a wide range of plants are affected by fungal interactions, but there have been few instances where that hypothesis has been directly tested.

Stable isotope natural abundance analysis is a useful approach to the study of mycoheterotrophic nutrient pathways (e.g., Gebauer and Meyer, 2003; Trudell et al., 2003; Ogura-Tsujita et al., 2009). Heterotrophically derived nutrients reflect the isotopic composition of their source and, because fungal C and sometimes nitrogen (N) are typically enriched in heavy isotopes relative to photosynthetically fixed carbon and soil-derived inorganic nitrogen, C and N isotopes can be used to estimate the degree of mycoheterotrophy (e.g., Gebauer and Meyer, 2003; Zimmer et al., 2007; Suetsugu et al., 2019). The carbon and nitrogen isotopic distinctiveness of nutrient contributions from saprotrophic fungi is far less than for fungi that simultaneously form ectomycorrhizal associations. Hydrogen isotopes are now being used to overcome the limited power of C and N isotopes to quantify mycoheterotrophy and have demonstrated greater fungal contribution to plant nutrition than previously suspected (Gebauer et al., 2016), but these methods are still not widely applied, and they were unavailable when the reported study was conducted. If the amount of mycoheterotrophy changes with environmental conditions or stress, then plant isotopic enrichment would be expected to reflect that change. Such changes in the relative contribution of fungi to plant nutrition and isotopic composition could result from increasing fungal contribution, decreasing photosynthetic contribution, or both (Jacquemyn et al., 2021). Additionally, isotopic composition can shift with direct effects of environmental conditions on stomatal conductance and photosynthesis.

We tested the hypothesis that photosynthetic orchids increase reliance on mycorrhizal fungi for C and N acquisition during periods of resource limitation. We used C and N isotope analysis to determine whether two orchids with different life history characteristics, *G. pubescens* and *Tipularia discolor*, relied more on mycorrhizal fungi for N and C under conditions of light limitation and decreased water availability. Light availability has been previously shown to influence photosynthesis in *Tipularia discolor* (Tissue et al., 1995; Hughes et al., 2019) and carbon acquisition in a species of *Goodyera* (Liebel et al., 2015). Both direct effects of drought on plants and increased fungal contribution to plant carbon would be expected to increase plant $\delta^{13}C$, but only increased fungal contribution would be expected to increase enrichment in ^{15}N. We hypothesized that increased shade would decrease photosynthesis without directly affecting fungi, resulting in orchid leaves enriched in ^{13}C, reflecting increased fungal contribution to plant carbon. In contrast, we hypothesized that drought would affect photosynthesis through decreased stomatal conductance but would potentially decrease fungal contribution to plant carbon. We expected the two species to differ in mean isotopic enrichment, because they associated with different fungi and were active at different times of the year, but we expected isotopic enrichment to increase or decrease similarly in response to treatment conditions.

Materials and methods

Study species

Goodyera pubescens R.Br is an evergreen orchid occurring in mid and late successional forests throughout eastern United States. Individual plants have a basal rosette of leaves with new leaves produced in the spring. Flowering occurs in mid-summer and the inflorescence emerges from the center of the basal rosette. After flowering, rhizomes may branch, allowing limited asexual reproduction, but clones remain small and did not extend beyond the experimental treatments. Pelotons of mycorrhizal fungi are present year-round in older roots and colonize newly produced roots (Rasmussen and Whigham, 2002). Plants associate exclusively with a single clade of *Tulasnella* spp. (McCormick et al., 2004) that decompose organic matter as their primary form of nutrition, and can switch fungi following drought (McCormick et al., 2006).

Tipularia discolor (Pursh) Nutt. is a winter-green orchid that produces a single leaf that appears in early autumn, typically September-October, and senesces in the spring, typically May. Flowering occurs at the end of July or beginning of August when leaves are not present. The species occurs primarily in hardwood forests throughout Eastern US. Fungal pelotons are present in roots throughout the year, with two fungi in the genus *Protomerulius* that support seed germination and protocorm growth. After becoming photosynthetic, the species associates with a wide range of fungi that belong to several distantly related *Tulasnella* clades (McCormick et al., 2004).

Study location

The experiments were conducted in six deciduous forest stands, three for each species of orchid, at the Smithsonian Environmental Research Center (SERC) in Edgewater, Maryland, USA. For both orchids, in each site we located 12 mature plants. For *G. pubescens*, the plants had rosettes that were ≥4 cm diameter and ≥five leaves. *Tipularia discolor* plants, which produce a single leaf per year, had leaves that were ≥3 cm wide. The orchids that were selected in each forest stand were separated by 1–2 meters to prevent sampling multiple plants associated with a single fungal organism. McCormick et al. (2006) found that orchids separated by more than 50 cm associated with different fungal individuals. We randomly assigned four individuals of each species to the shade and drought treatments (described below) and controls. We also selected 12 *Fagus grandifolia* Ehrh. seedlings (10–20 cm tall) associated with each selected orchid to serve as autotrophic reference plants. *Fagus grandifolia* was the only autotrophic species present across all study sites and <20 cm away from each selected orchid.

Experimental set-ups

Individual orchids subjected to drought or shade treatments were covered by exclosure shelters, each constructed of 1.9 cm diameter PVC pipe to form a 50 cm × 50 cm canopy with 28 cm legs. Shade structures used black 95% shade cloth as per Gorchov et al. (2011). Drought shelters were covered with UV-permeable rain barrier plastic (2-mil ACLAR 22A, Honeywell Specialty Films, Linden, NJ, USA), and were bordered on their uphill edge by a 50 cm length of landscape edging to divert surface runoff. Shelters for *G. pubescens* remained in place from June-October 2009 (5 months) and shelters over *T. discolor* remained in place from October 2009–February 2011 (16 months). Leaves of both species were collected for isotope analysis, described below, at the end of the study. In addition to the two orchids and reference *F. grandifolia* seedlings, we also collected leaves for isotope analysis from two seedlings of *F. grandifolia* that were growing beneath two of the *T. discolor* exclosures, one beneath a shade and the other beneath a drought treatment. While just two *F. grandifolia* seedlings make for a very small sample size, we had hoped to have far more *F. grandifolia* individuals, as well as other species, but few other plants grow in the shaded understory locations where the study took place and no other plants survived the treatments.

Environmental data

Soil moisture data associated with each shelter were collected at the beginning and end of each experiment to assess relative differences among sites, and to verify treatment efficacy. Percent soil moisture between 0 and 12 cm depth was measured at eight locations in each site to account for site differences in water availability (Hydrosense^{TM} Moisture Meter, Campbell Scientific Australia Pty. Ltd., Garbutt, QLD, Australia). Photosynthetically active radiation (PAR) was measured using an AccuPAR PAR-80 1 m Sunfleck ceptometer (Decagon Devices, Pullman, WA, USA) at 50 cm above the ground, a height that reflected light levels that plants would have been experiencing prior to manipulation, at each plant location, and at random locations within each forest stand. PAR was highly variable (range 1–46% available PAR) among locations, sites, and beneath the light shelters and was never a significant factor in plant C, N, or isotopic enrichment (all $P > 0.6$), so it was not included in final analyses. Soil moisture values were compared among treatments (fixed independent variable) and sites (random independent variable) using an ANOVA. Experimental treatment and site effects were analyzed using two-way ANOVAs in Systat v 12.0 with site and treatment as main effects and interactions. Where treatment effects were significant, we conducted a *post-hoc* comparison among the treatment means using Tukey's honestly significant difference test in Systat (12.0).

Plant growth and ecophysiology

For *G. pubescens*, initial plant size was measured by taking a photograph of each plant with a ruler for scale. Photos were printed, using the ruler to check for scale, and each leaf was cut out and area measured using a LI-3100 area meter (LiCor, Lincoln, NE, USA). At the end of the experiment, plants were again photographed, and area measured as before. Growth of each plant was calculated as the change in area from the beginning of the experiment until the end.

For *T. discolor*, we measured the length and width of each leaf (each plant produced a single leaf per year) using a ruler and converted to area using: Leaf Area = 2/3 (length × width). Growth of each leaf was based on area at the beginning and end of the experiment. For this species, we took advantage of the availability of a LiCor instrument that was not available when we conducted the study with *G. pubescens*. We measured the effects of drought and shade on *T. discolor* light saturated rates of photosynthesis (A_{sat}) using a LiCor 6400 (LiCor Biosciences, Lincoln, NE, USA) on a warm (air temperature was 19.5°C) winter day, February 18, 2011. Hughes et al. (2019) found that February was when *T. discolor* had the highest rates of photosynthesis, reflecting a combination of increased light in the forest understory and physiological activity. A_{sat} was measured with the instrument set to the following conditions: block temperature: 25°C, PAR: 1,000 micromols, as these represent optimum light and temperature conditions for this species (Tissue et al., 1995). After a minimum of 5 min under light saturated condition, we began to log data.

We then changed PAR to 0 to estimate dark respiration (R_d) and waited a minimum of 3 minutes until steady state conditions were achieved prior to logging data. Water use efficiency (WUE) under light saturated conditions was calculated as A_{sat}/transpiration. We also performed rapid light curves using pulse amplitude modulated fluorometry (Mini PAM, Walz, Hamburg, Germany) to estimate quantum efficiency (α) and the maximum relative electron transport rate (rETR). Briefly, leaves were exposed to eight increasing levels of PAR for 10 seconds, followed by a 0.6 saturation pulse of light. Both α and rETR were fit in non-linear models in SAS (v 9.2) (proc nlin) as described by Ralph and Gademann (2005).

Experimental treatment and site effects on A_{sat}, R_d, α, and rETR were analyzed using two-way ANOVAs in Systat v 12.0 with site and treatment as main effects and interactions. Where treatment effects were significant, we conducted a *post-hoc* comparison among the treatment means using Tukey's honestly significant difference test in Systat (12.0).

Stable isotope abundances and nitrogen and carbon concentrations

Goodyera pubescens leaves were collected after 17 weeks of treatment and analyzed for relative isotopic abundance. We collected the youngest full-sized leaf from the center of each rosette. This ensured that we were collecting a leaf that had formed after the treatment was initiated, hence minimizing dilution of treatment effects through averaging over the lifespan of leaves that were already present when treatments began (e.g., Hynson et al., 2012). At the same time, we harvested the youngest full-sized leaves from the nearby *F. grandifolia* seedlings. After each leaf harvest, scissors used to cut each leaf were cleaned with 95% ethanol to prevent cross-contamination. Leaves were placed directly into sterile micro-centrifuge tubes, returned to the laboratory within 3 h, and stored at −20°C until they were prepared for isotope mass spectrometry (below).

Fully expanded leaves of *T. discolor* were collected in February 2011, 16 months after the experiment began. This ensured we were collecting leaves that initiated after treatment onset. The youngest full-sized leaves of the nearby *F. grandifolia* near the *T. discolor* shelters were collected earlier, October 2010. *Fagus grandifolia* leaves were collected at a different time because by the time *T. discolor* leaves were fully developed, the reference plants would not have had leaves. Leaves of the two *F. grandifolia* seedlings growing beneath *T. discolor* shelters (described above) were also sampled in October 2010.

Frozen leaf samples from the orchids and *F. grandifolia* were lyophilized, ground, and weighed (3–4 mg of ground foliar tissue) into 5 × 9 mm tin capsules (Costech Analytical Technologies, Valencia, CA, USA). Stable isotope ratio gas chromatography mass spectrometry (EA-IRMS) analyses were completed at the Smithsonian Museum Conservation Institute Stable Isotope/Mass Spectrometry Lab in Suitland, MD, using an Elemental Analyzer Model 4010 (Costech Analytical Technologies) coupled to a Delta V Advantage Isotopic Ratio Mass Spectrometer with Isodat NT Software (Thermo Fisher Scientific, Waltham, MA, USA).

Measured isotope abundances are presented as δ-values and calculated using the equation: $\delta^{15}N$ or $\delta^{13}C = (R_{sample}/R_{standard} - 1) \times 1000$ [‰] (where R_{sample} and $R_{standard}$ are ratios of heavy:light isotope of each element in the sample or standard) (Gebauer and Meyer, 2003). Because stable isotope composition is affected by local climatic conditions, relative isotope ratios were normalized to site-specific enrichment factors for each species using the equation: $\varepsilon = \delta x_S - \delta x_R$, where δx_S is the individual $\delta^{15}N$ or $\delta^{13}C$ value of a sample, and δx_R is the mean $\delta^{15}N$ or $\delta^{13}C$ of the 12 autotrophic reference plants at the site in question (Preiss and Gebauer, 2008). Total %N and %C were measured on all leaf samples with the same instrument used for the isotope analysis.

Percent N and C data were analyzed for correlations with isotopic composition, and to determine whether they varied with sample date or experimental treatment. We compared the isotopic enrichment of drought, shade, and control plants using ANOVAs with $\varepsilon^{15}N$ and $\varepsilon^{13}C$ as dependent variables. Treatment (control, shade, drought) and species were fixed independent variables, and site (nested within species) was a random variable. After considering the effect of treatment overall, we compared drought and shade treatments in a second set of identical ANOVAs. All calculations were conducted using Systat 12 for Windows (Systat Software Inc., San Jose, CA, USA).

Results

Environmental data

Soil moisture varied among sites and treatments for both species (Table 1). The treatment effect was significant ($F = 61.3$, $P < 0.001$). Soil moisture differed among the species, which was expected, since the studies were carried out in different years and seasons, and was significantly lower inside the precipitation exclosures for both species, compared to shade or control locations (ANOVA: Species: $F = 37.5$, $P < 0.001$; Treatment: $F = 61.3$, $P < 0.001$; Species × Treatment: $F = 2.05$, $P = 0.15$, Species (Site): $F = 15.6$, $P < 0.001$; Supplementary Figure 1). Shaded and control soils did not differ significantly ($F = 0.700$, $P = 0.41$); Species × Treatment: $F = 2.05$, $P = 0.15$, Species (Site): $F = 15.6$, $P < 0.001$.

TABLE 1 Percent soil moisture (volumetric ± se) at each of the three sites and three treatments (control, drought, and shade) for *Goodyera pubescens* and *Tipularia discolor*.

	Site 1	Site 2	Site 3
Goodyera pubescens			
Control	23.7 ± 5.4	21.3 ± 1.2	31.5 ± 1.8
Drought	12.2 ± 1.6	11.9 ± 1.1	18.0 ± 1.1
Shade	19.5 ± 1.5	22.3 ± 2.5	29.3 ± 1.8
Tipularia discolor			
Control	20.6 ± 1.2	19.4 ± 0.5	17.0 ± 0.2
Drought	11.6 ± 0.2	12.0 ± 0.2	11.2 ± 0.2
Shade	20.3 ± 1.2	18.9 ± 0.1	16.4 ± 0.5

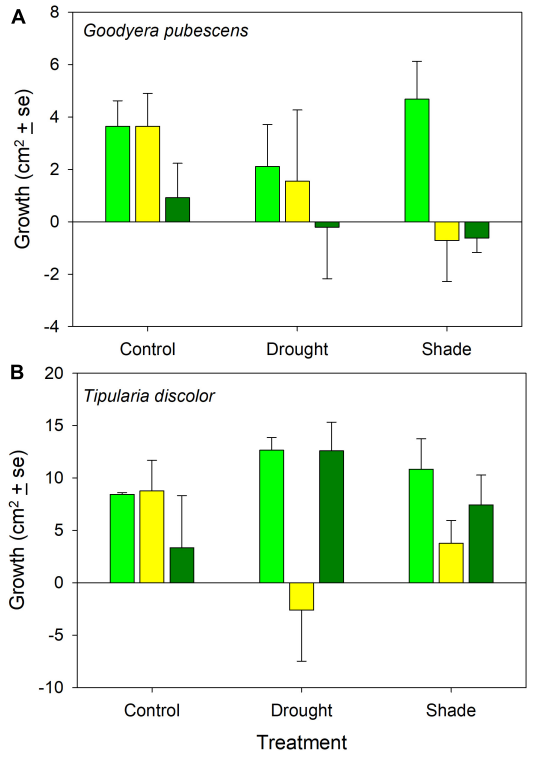

FIGURE 1
Relative leaf area growth (mean ± 1 SE) for **(A)** *Goodyera pubescens* and **(B)** *Tipularia discolor* control and treatment plots at each site. The three bars for the two treatments and controls represent different sites where the experiment was conducted, as described in the section "Materials and methods."

Orchid growth

Relative leaf growth was significantly different between species (Figures 1A,B; ANOVA: Species: $F = 13.5$, $P = 0.001$) but the treatment and interaction effects were not significant (Treatment: $F = 0.038$, $P = 0.96$; Species × Treatment: $F = 0.022$, $P = 0.98$, Species (Site): $F = 3.41$, $P = 0.014$). Relative leaf growth was positive in all of the controls and the majority of treatments

TABLE 2 Carbon (%C) and nitrogen (%N) concentrations (each mean ± se) of *G. pubescens* and *T. discolor* across all treatments (top two rows) and concentrations across treatments (bottom three rows).

	%C	%N
Species		
Goodyera pubescens	44.14 ± 0.20	2.08 ± 0.10
Tipularia discolor	44.46 ± 0.14	2.59 ± 0.09
Treatment		
Control	44.23 ± 0.20	2.37 ± 0.15
Shade	44.59 ± 0.18	2.52 ± 0.10
Drought	44.09 ± 0.23	2.13 ± 0.11

but there were noticeable differences in relative growth between sites within the two treatment sites (Figure 1A). Relative leaf growth was not different from zero, based on the size of the error bars, in one of the drought treatment plots, and negative in two of the shaded plots (Figure 1A).

Stable isotope abundances and nitrogen and carbon concentrations

There were no significant species, treatment, or sites effects for leaf C (Table 2; ANOVA: Species: $F = 1.81$, $P = 0.18$; Treatment: $F = 1.57$, $P = 0.22$), and the interactions between Species × Treatment ($F = 0.808$, $P = 0.45$) and Species (Site) ($F = 0.890$, $P = 0.48$) were not significant. Leaf N concentrations were significantly higher for *T. discolor* (Species: $F = 16.0$, $P < 0.001$) and drought-exposed plants had lower %N than control or shaded plants ($F = 3.35$, $P = 0.042$). The Species × Treatment ($F = 0.011$, $P = 0.99$) and Species (Site) ($F = 1.61$, $P = 0.18$) interactions were not significant for %N.

Drought and shade treated plants of both orchids were enriched in both ^{13}C and ^{15}N compared to controls (Figure 2), but the species differences were only significant for $\varepsilon^{13}C$ (Table 3). For both species, there were between plot differences in $\varepsilon^{13}C$ and $\varepsilon^{15}N$ (Figure 3), but the difference between shade and drought treated plants was not significant (both $P > 0.56$). Leaves of both orchids differed from the control *F. grandifolia* leaves (Figure 2). As described in the Methods, we were able to sample a single *F. grandifolia* seeding in a shaded and drought *T. discolor* plot. The shaded seedling had $\varepsilon^{13}C = -0.51$ and $\varepsilon^{15}N = 0.022$, and the drought-exposed seedling had $\varepsilon^{13}C = 0.53$ and $\varepsilon^{15}N = -0.06$ (Figure 2).

Ecophysiology

As expected, photosynthesis of *T. discolor* leaves differed between treatments, as demonstrated by significantly different

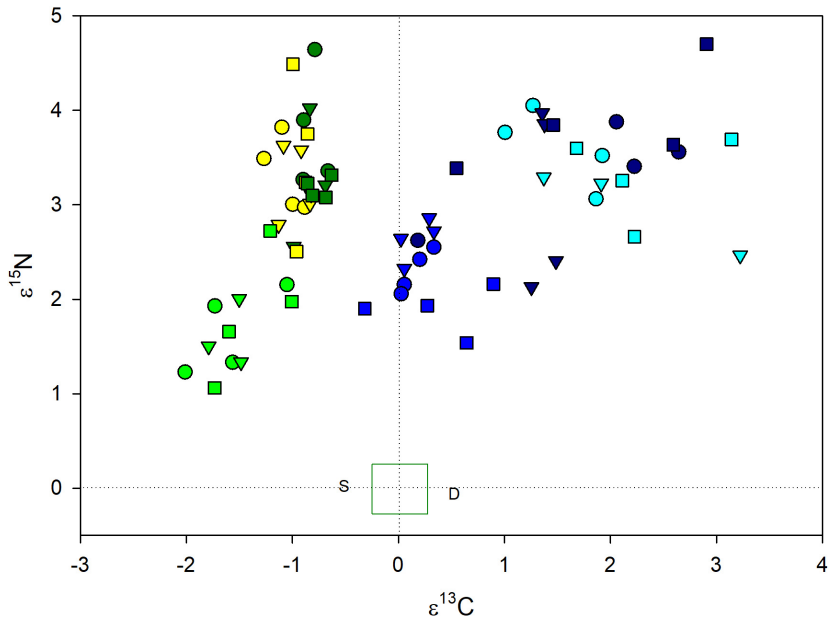

FIGURE 2
Isotopic enrichment factors for ^{13}C and ^{15}N orchids (*G. pubescens*: shades of green/yellow) and (*T. discolor*: shades of blue) exposed to control (medium green or blue), drought (yellow or light blue), and shade (dark green or dark blue) treatments. Within each species, values for orchids from the three sites are presented as different symbols, though overlapping symbol shapes for the two species do not indicate shared sites. The green box around 0,0 indicates the mean (defined as 0) and standard error of enrichment factors for autotrophic reference plants (*Fagus grandifolia*). The isotopic enrichment factors for the single surviving shaded and drought-treated *F. grandifolia* are indicated as S and D, respectively.

TABLE 3 The results of ANOVA tests for differences in carbon concentration (%C), nitrogen concentration (%N), and enrichment in ^{13}C and ^{15}N among orchid species, treatments (drought, shade, and control), sites within species, and species × treatment interactions.

	df	*F*	*P*
%C			
Species	1	1.81	0.18
Treatment	2	1.57	0.22
Species(site)	4	0.89	0.48
Species × Treatment	2	0.808	0.45
%N			
Species	1	16.0	<0.001
Treatment	2	3.35	0.042
Species(site)	4	1.61	0.18
Species × Treatment	2	0.011	0.99
$\varepsilon^{13}C$			
Species	1	394	<0.001
Treatment	2	30.1	<0.001
Species(site)	4	0.872	0.48
Species × Treatment	2	10.3	<0.001
$\varepsilon^{15}N$			
Species	1	2.13	0.15
Treatment	2	49.2	<0.001
Species(site)	4	0.379	0.28
Species × Treatment	2	2.00	0.14

quantum efficiency, α (F = 4.01, P = 0.04), and marginally significantly different rate of photosynthesis under light-saturating conditions, A_{sat} (F = 3.48, P = 0.09). Dark respiration (R_d) was highly variable and not statistically different across sites or treatments (**Table 4**). Control plants had higher mean rates of light-saturated photosynthesis (A_{sat}) than shaded or drought-treated plants (F = 8.93, P = 0.015), perhaps reflecting their lower stress level. Shaded plants had higher quantum efficiencies (α) than control and drought-treated plants (F = 7.07, P = 0.015), demonstrating the effects of light limitation. There were also significant differences among sites in rETR (F = 8.37, P = 0.002) and WUE (F = 4.25, P = 0.06) (**Table 4**).

Discussion

This study offers experimental data supporting the hypothesis that light and drought stress can increase orchid dependence on fungal carbon. Selosse and Roy (2008) and Motomura et al. (2010) hypothesized that increasing mycoheterotrophy leads to the evolution of achlorophyllous, totally mycoheterotrophic plants, and that this might be triggered by very low light conditions. Others have shown a correlation between light availability and plant isotopic concentrations, indicative of fungal contribution to plant carbon (e.g., Gebauer, 2005; Liebel et al., 2010; Preiss et al., 2010), and a recent study used multiple isotopes to demonstrate a previously difficult to discern connection between light levels and partial mycoheterotrophy (Schweiger et al., 2019). In contrast, Těšitel et al. (2018) provided an argument for the retention of photosynthesis in partially mycoheterotrophic plants, as a support for seed set. None of these studies demonstrated the shifting of photosynthetic contributions with a manipulative experiment. We found that drought and shade stresses caused declines in parameters related to photosynthesis and resulted in increased reliance on mycoheterotrophy. We propose that the increased mycoheterotrophic contribution to orchid nutrition facilitated the maintenance of similar growth rates in both species and dark respiration in *T. discolor* despite the stress conditions.

The increased reliance on mycoheterotrophy was interpreted from increased enrichment in ^{13}C and ^{15}N, but isotopic composition can also be affected by other factors. Both drought and shade resulted in enrichment in ^{13}C. However, both stressors can also have direct effects on ^{13}C composition. In the absence of a changed fungal contribution to carbon, we would have expected that a direct effect of shade on plant carbon cycling would led to depleted ^{13}C, as shown for a wide range of non-orchid autotrophs (Preiss et al., 2010; Liebel et al., 2015; Lallemand et al., 2018). This also appears to be borne out by the single shaded *F. grandifolia* seedling, which had a lower $\varepsilon^{13}C$ than the untreated autotrophic reference plants. Importantly, we found that the actual difference was in the opposite direction, demonstrating that the orchids were more enriched in ^{13}C, not less. The results suggest that our measurements may have underestimated the true increases in mycoheterotrophy that occurred as a result of shading.

In contrast to the shade treatment, we hypothesized that drought-treated orchids would be unable to increase the fungal contribution to C. However, we measured enrichment in ^{13}C in drought-treated orchids that was almost the same as for shade. Although we do not know the extent to which OMF are able to translocate water, a possible reason for not seeing the expected decrease in $\varepsilon^{13}C$ could be that the drought imposed by the shelters was too localized to affect the fungi, which might have been able to translocate water from outside the shelters. For example, Ruth et al. (2011) found that arbuscular mycorrhizal fungi were able to translocate water from one chamber to an associated plant in another chamber. Another possible explanation for the increase in $\varepsilon^{13}C$ could be the direct effects of drought. The $\delta^{13}C$ of plants experiencing drought has been found to increase 1–2‰ as a result of stomatal closure to minimize water loss and increased internal recycling of CO_2 (e.g., Pollastrini et al., 2010). However, the single *F. grandifolia* seedling that was exposed to drought was only slightly enriched in ^{13}C compared to untreated autotrophic control plants, suggesting that while our isotopic measurements might have led to an overestimate of increases in mycoheterotrophy in response to drought, they do not seem to negate them.

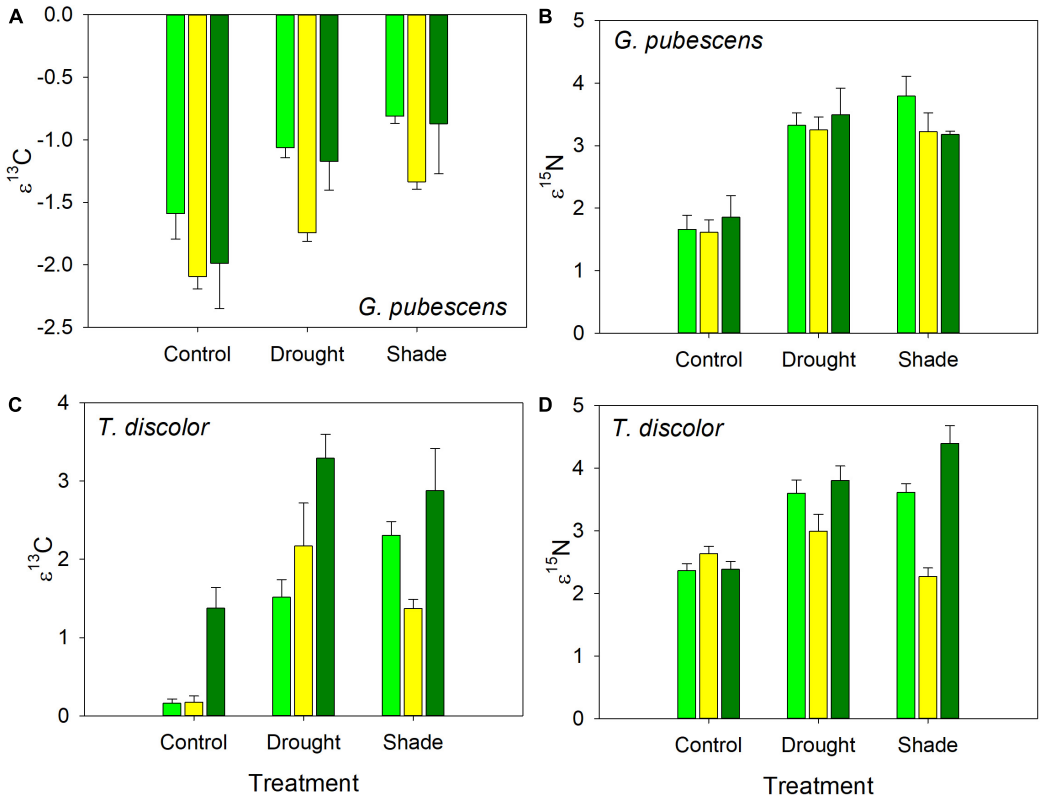

FIGURE 3
Isotopic enrichment factors for ^{13}C and ^{15}N for *G. pubescens* **(A,B)** and *T. discolor* **(C,D)** exposed to control, drought, and shade treatments. Mean enrichment factor ± se is shown. Different colored bars indicate different sites.

TABLE 4 Mean ecophysiological parameters (±se) for *T. discolor* in three sites under control conditions or exposed to drought or shade.

Site	Treatment	rETR	α	WUE	A_{sat}	R_d
1	Control	77.14 ± 5.87	0.28 ± 0.03	9.89 ± 0.98	6.78 ± 0.47	−0.71 ± 0.13
2	Control	58.55 ± 6.87	0.28 ± 0.02	34.73	5.87	−0.29
3	Control	92.23 ± 7.63	0.28 ± 0.01	14.6 ± 4	6.44 ± 1.75	−0.81 ± 0.08
1	Drought	71.72 ± 8.24	0.27 ± 0.01	9.22 ± 0.39	4.85 ± 0.29	−0.49 ± 0.08
2	Drought	47.31 ± 7.65	0.26 ± 0.02	22.69 ± 10.54	3.79 ± 0.3	−0.54 ± 0.05
3	Drought	103.20	0.23	11.36 ± 3.08	4.9 ± 1.59	−0.63 ± 0.11
1	Shade	83.03 ± 7.73	0.32 ± 0.01	9.00	3.32	−0.45
2	Shade	70.27 ± 6.83	0.3 ± 0.02	17.69 ± 0.56	3.68 ± 1.21	−0.87 ± 0.08
3	Shade	66.5 ± 10.62	0.31 ± 0.02	8.35 ± 0.21	4.51 ± 1.07	−0.54 ± 0.08

rETR, maximum relative electron transport rate; α, quantum efficiency; WUE, water use efficiency; A_{sat}, light saturated photosynthesis; R_d, dark respiration. For some plots, only one plant was available to measure, so the values for that plant are given with no standard error reported.

Another possible cause for the observed enrichment of ^{13}C is that drought could disrupt the orchid-fungus relationship and cause orchids to associate with different, perhaps more drought-resistant, fungi. McCormick et al. (2006) found that *G. pubescens* in locations near the present study sites switched to associate with different fungal genets following a drought. If such a switch happened in this experiment, the new fungi could have had different isotopic compositions than the original fungi, leading to shifts in orchid isotopic composition without corresponding changes in quantitative contributions to plant carbon. This is only a realistic possibility for *T. discolor* because it associates with diverse fungi that can include fungi that are ectomycorrhizal with surrounding trees, and so potentially isotopically very different (e.g., Gebauer and Meyer, 2003). However, in many *T. discolor* individuals sampled over the course of 10 years, we have not found any that were

sufficiently enriched in ^{15}N to suggest a considerable input from ectomycorrhizal fungi. In *G. pubescens*, fungal associates belong to a very narrow clade that we would expect to be isotopically very similar.

Patterns for ^{15}N enrichment mirrored those for ^{13}C, suggesting that both nitrogen and carbon were taken up from fungi, and that the uptake was affected by drought and shade stress. The strongest differences in ^{15}N enrichment that reflect the extent of mycoheterotrophy have been seen in plants associating with ectomycorrhizal fungi, which are typically enriched in ^{15}N relative to N obtained from inorganic nutrients in the soil (e.g., Hynson et al., 2013). Saprotrophic fungi often have less enriched ^{15}N abundance, depending on what they decompose to obtain nutrients, and so the nitrogen "signal" for fungal contribution to plant nutrition is weaker (Bidartondo et al., 2004; Martos et al., 2009; Schweiger et al., 2019). Indeed, the observed increase in $\varepsilon^{15}N$ was far less than what has been observed for orchids associated with fungi that are simultaneously ectomycorrhizal with other plants, but it was still a detectable increase (e.g., Schiebold et al., 2018). This is particularly important for interpreting the direct effects of drought and shade on plant photosynthesis, and thus $\varepsilon^{13}C$, because $\varepsilon^{15}N$ is expected to be unaffected by direct effects of drought (Peuke et al., 2006) and shade, yet we saw a similar increase in $\delta^{15}N$ in drought-exposed and shaded plants. Accordingly, the two surviving *F. grandifolia* plants in the drought and shade treatments both had $\varepsilon^{15}N$ of nearly 0. However, drought-treated orchids also had lower overall nitrogen concentration, suggesting that less total nitrogen was taken up by the plants and that perhaps the fungal contribution to drought-treated plants, while a proportional increase compared to unstressed control orchids, nevertheless could have been less than for shaded plants.

The differences in photosynthetic parameters for *T. discolor* shed some light on how drought and shade affected plant physiology and complemented isotopic results. In particular, we found lower light saturated photosynthesis (A_{sat}) in shade and drought stressed orchids; suggesting that photosynthetic rates were lower in stressed plants. Shaded plants were able to photosynthesize more efficiently (greater α), a common adaptation to low light levels, but this was likely not enough to make up for the greatly decreased light availability in this treatment. Other physiological parameters, WUE, and R_d, were unchanged, suggesting that *T. discolor* was able to maintain many aspects of their physiology, despite decreased photosynthetic ability. Both orchid species were also able to maintain similar growth rates despite shade and drought stress, perhaps pointing to the importance of increased mycoheterotrophy for surviving stressful conditions.

Our results suggest that increased mycoheterotrophy may be triggered by stresses that limit photosynthetic ability and not just by limited light. The effects of experimental treatments on plant $\varepsilon^{13}C$ and $\varepsilon^{15}N$ indicated that the orchids can increase reliance on carbon derived from fungi when photosynthetic capability was encumbered. Although it has previously been postulated that limited light availability could determine reliance on fungi for carbon (Gebauer, 2005; Hynson et al., 2009; Liebel et al., 2010; Preiss et al., 2010), this study provides experimental evidence that reduced light availability and drought can both increase mycorrhizal dependence in a partially mycoheterotrophic orchid. These results, combined with the evidence that photosynthesis contributes primarily to above-ground parts of partially mycoheterotrophic plants (Těšitel et al., 2018), provide the framework on which to build a more detailed understanding of the evolution of mycorrhizal associations. Further, with investigation of other green orchids and partially mycoheterotrophic plants, this promises to advance understanding of the dynamics of mycoheterotrophy in forest understories, and may help to explain why nearly all forest understory herbs are mycorrhizal (Whigham, 2004), despite very low light availability, which would be expected to limit the availability of photosynthetic carbon to contribute to a bidirectional mycorrhizal association.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

MM and KG conceived the bulk of the study with additions by DW and TM. KG (2009–2011), MM (2009–2022), and TM (2011–2022) carried out the study. MM analyzed the data. MM and KG wrote the manuscript. DW and TM edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Host tree species effects on long-term persistence of epiphytic orchid populations

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The destinies of epiphytic orchids (about 70% of all orchids) are linked to their host trees. However, there is little information on if differences in host trees characteristics can affect the long-term persistence of orchid populations, and how this might vary under different climatic conditions. We compared the population dynamics of two epiphytic orchid species, *Alamania punicea* and *Oncidium brachyandrum* growing on two host trees with contrasting leaf phenologies: the deciduous *Quercus martinezii* and the semideciduous *Q. rugosa*, over 3 years with varying levels of rainfall, in a montane tropical oak forest in Oaxaca, Mexico. Using data from > 500 individuals growing on 63 host trees, we applied linear mixed effects models, Integral Projection Models, and Life Table Response Experiments to identify the effects of host tree on orchid vital rates and population growth rates. For both orchid species, survival and growth did not differ between host species during wettest year. However, during the driest year both vital rates were higher on the semi-deciduous host *Q. rugosa* than on the deciduous *Q. martinezii*. Host species did not affect fecundity for *A. punicea*, but for *O. brachyandrum* fecundity was higher on the deciduous host. For *A. punicea*, λ values were similar between hosts during the wettest and intermediate years, but significantly lower (Δ λ = 0.28) on the deciduous than on the semi-deciduous host during the driest year. This was due primarily to lower survival on the deciduous host. For *O. brachyandrum*, λ was slightly higher (Δ λ = 0.03) on the deciduous than the semideciduous host during the wettest year, due to higher growth and reproduction. However, during the intermediate and driest years, λ values were significantly higher on the semi-deciduous than on the deciduous host (Δ λ = 0.13 and 0.15, respectively). This was due to higher survival and growth. *A. punicea* populations appear more vulnerable to dry conditions than *O. brachyandrum,* likely due to its smaller pseudobulbs, and hence lower water-storing capacity. Our results show that host tree species can both influence the vital rates and the long-term dynamics of orchid populations,

and these effects vary across orchids species and over time. Our results highlight the importance of maintaining a diversity of host trees to ensure long-term population persistence.

KEYWORDS

Orchidaceae, population dynamics, host preference, integral projection models, life table response experiment, *Quercus*, *Oncidium*, *Alamania*

Introduction

The orchid family is comprised of 850 genera with more than 30,000 species, and 50% of these species are concentrated in tropical areas of the world (Chase et al., 2015). In Mexico, there are approximately 1,200 species of orchids. Nearly 40% of Mexican orchids are endemic. Some species of Mexican orchids, including epiphytes, are very attractive to horticulturists, collectors, and public, and are extracted from their natural habitats and sold illegally, which can lead to them being threatened or extirpated (Halbinger and Soto, 1997; Merritt et al., 2014).

For vascular epiphytes, the presence of their host trees is essential for the establishment and permanence of their populations. However, due to differences in host traits, not all trees offer the same conditions for the establishment and development of epiphytic orchids (Wagner et al., 2015). For example, rough bark texture can affect the capture of seeds [rugose and scaly barks favor seed adherence compared to smooth barks (Adhikari and Fischer, 2011; Gowland et al., 2013; Timsina et al., 2016)], while an ability to retain and release water can favor the germination of seeds [barks with higher water retention capacity and slower release rates favor seed germination (Callaway et al., 2002; Einzmann et al., 2015)]. Similarly, the presence of allelopathic compounds in the bark of trees can limit seed germination and establishment of epiphytes (Callaway et al., 2002; Harshani et al., 2014); the rate of bark exfoliation and the fragility of branches can lead to differential mortality rates as a result of epiphyte falls (López-Villalobos et al., 2008); and the nutrient quality of stemflows and throughfalls could affect growth and fertility of the epiphytic orchids (higher amounts of nutrients could increase growth and fecundity rates). Finally, the leaf phenology of the host trees can affect the demography of epiphytes (Einzmann et al., 2015; Ticktin et al., 2016). In addition, these and other host trees characteristics can not only influence epiphytes directly, but also indirectly by providing different microclimatic conditions for the mycorrhizal fungal community, which are indispensable for the germination of the orchid seeds. Different trees may possess different communities of fungi that may or may not favor the germination of orchids (Otero et al., 2007; Rasmussen et al., 2015).

Although there is a large literature on the effects of host traits on orchid germination, establishment and survival, there is little information on whether differences in vital rates may scale up across the life cycle to differentially affect population persistence. Similarly, if and how these differences may shift with climatic conditions is largely unexplored. For example, annual variation in climatic conditions can influence the demographic behavior of epiphytes (Mondragón et al., 2004; Ticktin et al., 2016). Populations of epiphytes growing on host species that allow for higher humidity due to their phenology, architecture or bark water holding capacity might perform better during dry years, but not during average or wettest years. The one epiphyte study that has assessed this showed that the vital rates and population growth rates of an epiphytic bromeliad were different when growing on perennial pine vs. deciduous oak host trees (Ticktin et al., 2016). Populations on oaks had higher fecundity, but those on pines had higher survival and growth. Growth rates of populations on both host genera increased with increasing dry season rainfall, but the effect was larger for populations on oaks. The authors concluded that the presence of both pine and oak trees is very important for long-term conservation of these bromeliad populations.

Although we are unaware of other studies that have addressed this question for orchids, it is of great importance for developing conservation and management plans. Like many other wild species, populations of orchids are threatened by habitat loss and conversion to monocultures of timber species or other types of plantations (Boelter et al., 2011; Mondragón Chaparro et al., 2015), where diversity of host tree species is considerably diminished, for such as reforestation with pine species only (Jiménez-Bautista et al., 2014), or substitution of native shade trees from coffee or cocoa plantations by *Inga* spp. trees (Peeters et al., 2003; Valencia et al., 2016). These changes, in combination with changes in distribution of preferred hosts of species of epiphytes due to climate change (Hsu et al., 2012), could present problems for the long-term persistence of epiphytic orchid populations.

We carried out a demographic study to provide the first test of whether and how host species can affect the population dynamics of epiphytic orchids. We focus on two epiphytic orchid species *Alamania punicea* Lex. in La Llave and Lex. and *Oncidium brachyandrum* Lindl, growing on two congeneric

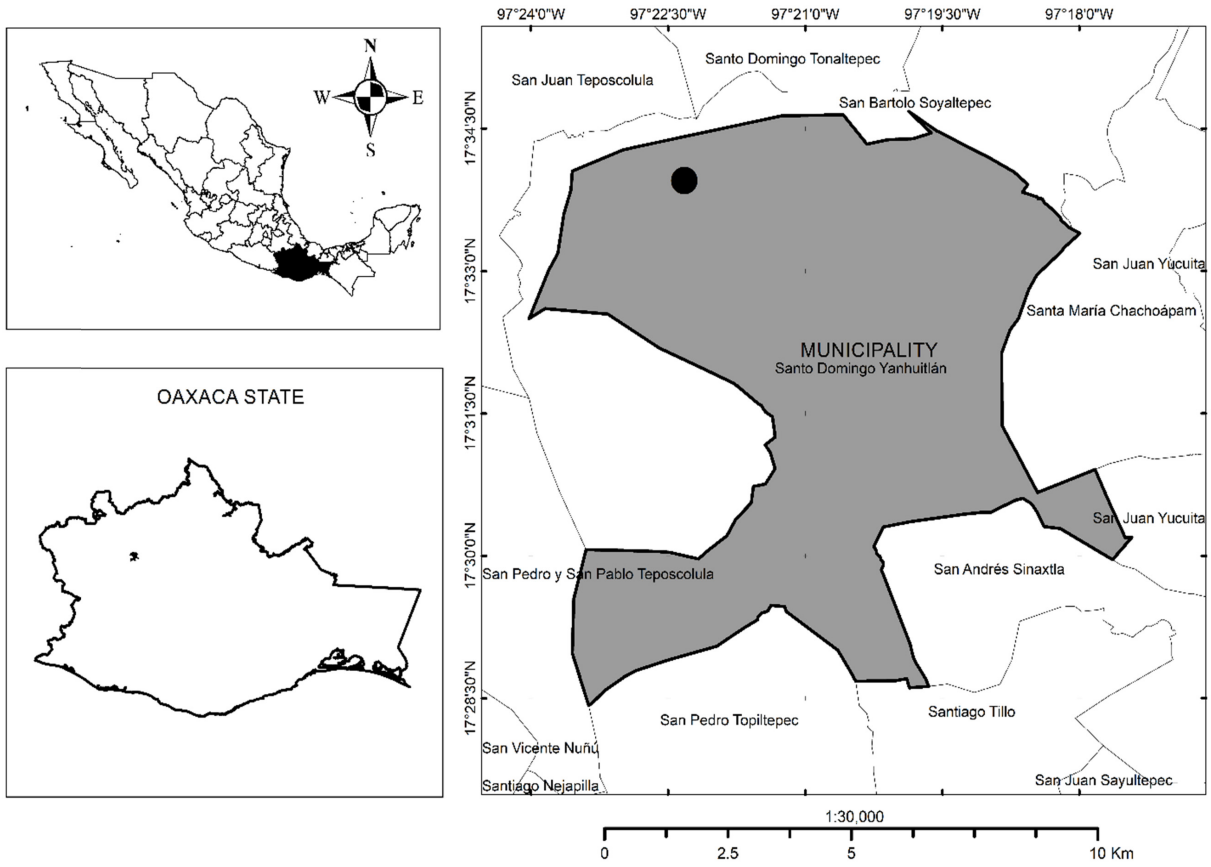

FIGURE 1
Geographic location of the study area within a seasonal oak forest in the municipality of Yanhuitlán, Oaxaca, Mexico.

host trees: the fully deciduous *Q. martinezii* Neé and the semi deciduous *Quercus rugosa* Neé. We addressed the following questions:

i) Do orchid vital rates (survival, growth, reproduction) vary between host tree species?
ii) Do differences in vital rates translate into differences in population growth rates?
iii) Do differences in vital rates and population growths rates vary with inter-annual climatic variation?

We hypothesized that:

(1) Survival and reproduction would be higher for both orchids on semi-deciduous *Q. rugosa* than fully deciduous *Q. martinezii*, due to lower light penetration and higher humidity in their treetops of the former, which can help orchids avoid photoinhibition (de la Rosa-Manzano et al., 2014; Einzmann et al., 2015) and increase flowering (Cervantes et al., 2005). In addition, we expected that branch fall, one of the main causes of mortality in epiphyte populations (Mondragón Chaparro et al., 2015; Cortes-Anzures et al., 2017), would be lower on *Q. rugosa* due to its thicker branches.
(2) Higher survival of individuals on the semideciduous *Q. rugosa* would translate into higher population growth rates (λ values), since population growth rates of long-lived species are highly sensitive to differences in survival (Franco and Silvertown, 2004). We also expected that the difference in λ values may be greater in drier years than in wettest years.

Materials and methods

Study area and species

This study was carried out in an oak forest in Tooxi, municipality of Yanhuitlán, Oaxaca, Mexico located in the Sierra Madre del Sur physiographical province (17°33′57.34″ N and 97°22′19.28″ W, elevation 2,579 m a.s.l; **Figure 1**) that encompasses the Mixteca Alta UNESCO Global Geopark

TABLE 1 Precipitation patterns in Tooxi, Yanhuitlán, Oaxaca from 2018 to 2020.

Year	Annual precipitation (mm)	Dry season (November–April) total/ average dry season monthly precipitation (mm/month)	Range: minimum/month-maximum/month during dry season (mm/month)
2018	1058	264.8/44.1 ± 24.2	14.9–84.7
2019	947	103.1/17.28 ± 4.3	10–22.3
2020	1041	125.2/20.9 ± 15.1	4.3–48.2

(García-Sánchez et al., 2021). Mean annual precipitation is 753 ± 152 mm, with a dry season average of 86 ± 67 mm. The mean temperature of the dry season is 14.4 ± 1.3°C, and the average maximum and minimum temperatures are 23.3 ± 1.8°C and 7.0 ± 2.9, respectively (INIFAP, 2021; **Table 1**). Tree vegetation includes deciduous, semi-deciduous and evergreen trees and is comprised mainly of *Quercus candicans* Neé, *Q. castanea* Neé, *Q. crassifolia* Humb. and Bonpl., *Q. rugosa* Neé, *Q. martinezii* C.H. Müll., *Juniperus flaccida* Schltdl., and *Arbutus xalapensis* Kunth. The epiphytic vegetation includes *Tillandsia bourgaei* Baker, *T. macdougallii*, *T. plumosa* Baker, *T. prodigiosa* (Lem.) Baker, *T. recurvata* (L.) L., *T. usneoides* (L.) L., *Pleopeltis conzatti* (Weath.) R. M. Tryon and A.F.Tryon, *Polypodium martensii* Mett., *Echeveria nodulosa* (Baker) Otto, *O. brachyandrum* and *A. punicea*.

We selected two species of tree hosts, *Q. rugosa* and *Q. martinezii* (**Table 2**) for this study. *Quercus rugosa* is distributed from Texas and Arizona, in the USA, to the Sierra Madre de Chiapas in Guatemala. Its populations are abundant in mountainous areas of western and central Mexico in temperate sub-humid climate, between the 1,800 and 2,800 m a.s.l. *Quercus martinezii* is endemic to Mexico and distributed in the states of Aguascalientes, Guanajuato, Hidalgo, Guerrero, Jalisco, Nuevo Leon, Nayarit, Puebla, Queretaro, San Luis Posoti, Tamaulipas, Veracruz, and Oaxaca from 2,000 to 2,500 m a.s.l (Valencia-A, 2004). The other species that hosted orchids (*Q. crassifolia* and *Q. candicans, J. flaccida* and *A. xalapensis*) had few individuals on them.

We focused on the two most abundant orchid species out of the three present at the study site. In addition, *Alamania punicea* is listed in CITES Appendix II, is the only species of a monotypic genus endemic to Mexico, and nothing is known about the demography of this species that could help to establish management and conservation strategies. *Oncidium brachyandrum* is harvested commercially species in Tlaxiaco, Oaxaca which is close to the study site (Ticktin et al., 2020). Nothing is known about the demography of this species; there is only one demographic study on another species of the genus (*Oncidium poikilostalix* Kraenzl.) M.W. Chase and N.H. Williams but in coffee plantations. We describe each orchid species as follows:

Alamania punicea Lex. is an epiphytic perennial orchid, 3–6 cm high including the inflorescence; ovoid pseudobulbs, slightly elongated, covered by translucent papyrus sheaths, 7–10 mm long; leaves 2, rarely 3, at the apex of the pseudobulb, elliptic to oblanceolate sheets, 1–4 cm long, 5–10 mm wide; flowers 7–14, red to pinkish reddish. Fruits are capsules with dust-like seeds. There is no report of *A. punicea* breeding system. Stpiczyńska et al. (2005) suggest that given its floral morphology, it could be pollinated by hummingbirds. *Alamania punicea* is an endemic species prevalent in cool and seasonally dry *Quercus-Pinus* forests on the *Trans*-Mexican Volcanic Belt and the Sierra Madre Oriental above 1,900 m a.s.l. (where oaks are dominant; García-Cruz et al., 2003; Soto Arenas, 2005; UNEP-WCMC, 2020).

Oncidium brachyandrum is an epiphytic perennial orchid, up to 20 cm high with clustered pseudobulbs, ovoid to ellipsoid or subglobose, somewhat laterally compressed, 2–3 cm long; 2 or 3 lateral leaves; flowers 2 or 3, simultaneous, showy, 25–30 mm in diameter, sepals, and petals brown or yellow with irregular brown spots and yellow lip. Fruits are capsules with dust-like seeds. This species probably is pollinated by oil-collecting or bombini bees and might be self-incompatible as reported for other members of the genera (Damon and Cruz-López, 2006; Pemberton, 2008). This orchid is distributed in Honduras, Guatemala, and in Mexico in the states of Durango, Guerrero, Jalisco, Michoacán de Ocampo, Morelos, Nayarit, Sinaloa, Zacatecas, and Oaxaca. It grows mainly in oak forests elevations of 2,000–2,500 m a.s.l. (Jiménez et al., 1998).

Precipitation, the most limiting factor for epiphytes (Zotz and Hietz, 2001; Laube and Zotz, 2003), varied across our three study years, especially during the dry season (**Table 1**). Although precipitation in all 3 years was above the mean of the past 20 years (**Supplementary material**), we refer to these years as the wettest year (2018), driest year (2019) where dry season precipitation was less than half that of the wettest year–and intermediate year (2020).

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TABLE 2 Host traits of two *Quercus* species in a seasonal oak forest in Yanhuitlán, Oaxaca, Mexico.

Trait	*Quercus martinezii*	*Quercus rugosa*
Leaf phenology	Deciduous	Semi-deciduous
Leaf area (cm^2)*	39.96 ± 4.61^{b}	59.70 ± 11.94^{a}
Tree height (m)[§]	9.69 ± 1.43^{a} ($n = 21$)	7.74 ± 2.05^{b} ($n = 42$)
Diameter at breast height (cm)	36.11 ± 12.59^{ns} ($n = 21$)	29.09 ± 17.72^{ns} ($n = 42$)
Bark roughness (cm)[†]	5.87 ± 0.39^{ns} ($n = 10$)	5.53 ± 0.59^{ns} ($n = 10$)
Water holding capacity of bark (ml/cm^3)	0.28 ± 0.07^{ns} ($n = 5$)	0.33 ± 0.09^{ns} ($n = 5$)
Canopy openness (%)		
Dry season	30.74 ± 6.74^{ns}	33.10 ± 8.91^{ns}
Wettest season	38.07 ± 5.81^{ns}	38.07 ± 2.24^{ns}
Relative humidity (%)		
Dry season	75.49 ± 15.96^{ns}	80.38 ± 14.04^{ns}
Wettest season	71.44 ± 10.32^{ns}	76.99 ± 9.30^{ns}
Mean temperature (°C)		
Dry season	14.17 ± 0.57^{ns}	14.52 ± 0.95^{ns}
Wettest season	13.76 ± 1.56^{ns}	13.47 ± 1.79^{ns}
Concentration of phosphorus in throughfalls (mg/l)	0.16 ± 0.03^{ns} $n = 5$	0.15 ± 0.05^{ns} $n = 5$
Concentration of potassium in throughfalls (mg/l)	2.56 ± 1.06^{ns} $n = 5$	2.76 ± 1.16^{ns} $n = 5$

Values are means ± SD for each tree species. Superscript lower-case letters indicate significant differences (ANOVA: $P < 0.05$, Tukey HSD).
[§]Significant differences across tree species [$F_{(1,62)} = 15.30$, $p = 0.0002$].
*Significant differences across tree species [$F_{(1,39)} = 47.52$, $p = 0.0001$].
[†]Information taken from Hernández-Álvarez (2021).

We selected 21 *Q. martinezii* and 42 *Q. rugosa* trees within a 1 ha plot and tagged and measured all the orchids of our study species on them. We selected a higher number of *Q. rugosa* trees since they had lower densities of orchids: on average there were 14 ± 11 *O. brachyandrum* plants/tree on *Q. martinezii* versus 6 ± 6 on *Q. rugosa*; these values were 8 ± 6 plants/tree vs. 7 ± 6, respectively, for *A. punicea*. For each of 3 years (2017–2020), we recorded plant status (alive, dead), size, and fecundity (number of capsules), and recorded the number of new seedlings. For both species of orchid, we measured height and width of the largest pseudobulb and counted the number of pseudobulbs. We also recorded causes of mortality distinguishing broadly between desiccation (the entire dead plant was still attached to the tree) and falling (the plant was missing). Although individuals that die due to desiccation are susceptible to falling, our monthly checks ensured that we were able to ascertain the correct cause of mortality. We did not include herbivory as a cause of death since we did not observe orchid plants attacked by herbivores at our study site. This is consistent with findings from other studies that rates of herbivory are mostly low in epiphytes (Benzing, 1990; Zotz, 1998; Winkler et al., 2005). Finally, we measured host characteristics on a subsample of individuals of each host species and tested for differences between hosts using ANOVAs (**Table 2**; Ramírez-Martínez, 2022).

Data analyses

Host effects on demographic rates (vital rates)

We tested differences in survival, growth, and reproduction of orchid individuals on the different *Quercus* species using generalized linear mixed models (GLMMs). Initial size (log-transformed), host species (*Q. martinezii* vs. *Q. rugosa*), and year were fixed effects and individual orchid nested within individual host tree were random effects. We used regression analyses to identify which measure of size (e.g., number of pseudobulbs, size of the pseudobulb, etc.) was the best predictor of growth, reproduction, and survival. We used Akaike's information criterion (AICc) to compare model fit. We found that for *A. punicea*, the best predictor was an index of number of leaves times area of the longest leaf (calculated with the formula for an oval). For *O. brachyandrum,* the best predictor was pseudobulb area (calculated with the formula for an oval).

To model the probability of survival, reproduction, and probability of mortality due to desiccation, we used binomial distributions, and to model the number of capsules, we used a negative binomial model. To model growth (size at $t + 1$), we used Gaussian error structure with an exponential variance structure, where the variance increases as an exponential function of initial size (Zuur et al., 2009). We modeled the probability of reproduction with the minimum size observed for plant reproduction (sizes: *A. punicea* ≥ 1.44 cm^2, and *O. brachyandrum* ≥ 0.79 cm^2). We selected the best fit model based on the lowest Akaike (AICc). All analyses were performed using the glmmTMB package in R v. 4.1.1.

Host effects on population growth dynamics

Integral projection models

We used integral projection models [IPMs (Easterling et al., 2000)] to project the long-term (asymptotic) population growth rates (λ values) of each orchid species growing on each of the two host species. The IPMs are constructed from continuous functions that describe size-dependent growth, survivorship, and fecundity. The IPM kernel is the sum of two functions. One describes the survival, probability and growth (or shrinkage) of survivors (p kernel), and the second is the reproductive contribution of each individual and the size distribution of the new seedlings (f kernel). Our IPM took the form:

$$n(y,\ t+1) = \int_{L}^{U} [p(x,\ y) + f(x,y)]n(x,\ t)\,dx$$

For both orchid species, the $p(x,y)$ kernel was represented by the survival probability of individuals of size x to size y attributable to size-dependent survival, s(x), and growth $g(x,y)$: $p(x,y) = s(x)\ g(x,y)$. The fertility kernel $f(x,y)$ denotes the production of new seedlings of size (x) produced from plants of size (y). This was calculated for plants of reproductive size as: $f(x,y) = s(x)\ f_n(x)\ p_E f_d(y)$, where $s(x)$ is the survival of plants of size (x), $f_n(x)$ is the probability of producing capsules for plant size x times the number of capsules per plant size x; p_E is the number of new seedlings produced per capsule, and $f_d(y)$ is the size distribution of new seedlings. For each host species, p_E was calculated as the number of seedlings observed in the field divided by the total number of capsules produced. We calculated the asymptotic projected population growth rate (λ) for each IPM using the popbio 2.7 package in R (Stubben and Milligan, 2007) and obtained 95% confidence intervals by bootstrapping ($N = 100$).

We used life table response experiments (LTREs) (Caswell, 2001) to identify which vital rate contributed most to the observed differences in population growth rates between hosts, for each orchid species and year.

Results

Host effects on vital rates

Alamania punicea

Survival

For individuals on both host species, survival increased as a function of size and was highest during the wettest year and lowest during the driest year (Table 3 and Figure 2A). The best fit model included an interaction between host species and year such that difference in survival between individuals on semideciduous *Q. rugosa* and the deciduous *Q. martinezii* was least during the wettest year. The probability of mortality due to desiccation decreased significantly with (log) size ($\beta = -0.69$, SE = 0.09, $z = -7.35$, $p < 0.001$) but did not differ significantly between host trees.

Growth

For individuals on both species, growth was lowest in the driest year and highest in the intermediate year. During the dry year, growth was higher on the semi-deciduous *Q. martinezii* than on the deciduous *Q. martinezii* (Table 3 and Figure 2B).

Reproduction

Plants began to reproduce once they reached 1.08 cm^2 size (number of leaves times area of the longest leaf). Only 1.4% of plants flowered and 100% those produced capsules. The probability of reproduction increased as a function of size, as did the number of capsules produced by reproducing plants (Table 3).

TABLE 3 Estimated coefficients from mixed-effect models of the probability of survival, growth, reproduction, and probability of producing capsules for *Alamania punicea* plants growing on two *Quercus* species.

Fixed effects	Estimate	SE	Z value	P-value
Probability of surviving to $t + 1$[†]				
Intercept	1.6513	0.4654	3.548	0.000388
Size at start	0.5076	0.1049	4.837	$1.32e-06$
Year 2 (2018–2019)	−1.3216	0.4157	−3.179	0.001477
Year 3 (2019–2020)	−1.0069	0.4871	−2.067	0.038704
Host species (*Q. rugosa*)	0.4296	0.6309	0.681	0.495931
Year 2 × *Q. rugosa*	1.1342	0.6219	1.824	0.068194
Year 3 × *Q. rugosa*	−0.5589	0.6066	−0.921	0.356817
Size at $t + 1$ of surviving individuals (growth)[§]				
Intercept	0.30439	0.10874	2.80	0.00512
Size at start	0.83544	0.02640	31.65	< 2e-16
Year 2 (2018–2019)	−0.52526	0.11704	−4.49	$7.2e-06$
Year 3 (2019–2020)	0.39822	0.12676	3.14	0.00168
Host species (*Q. rugosa*)	−0.06609	0.12641	−0.52	0.60110
Year 2 × *Q. rugosa*	0.33882	0.16004	2.12	0.03425
Year 3 × *Q. rugosa*	−0.11524	0.17310	−0.67	0.50557
Probability of producing capsules at time t (for individuals ≥ 35 cm)[†]				
Intercept	−4.8208	0.7344	−6.564	$5.23e-11$
Size at start	0.5040	0.1692	2.980	0.00289
Year 2 (2018–2019)	0.5833	0.6006	0.971	0.33141
Year 3 (2019–2020)	0.4047	0.7060	0.573	0.56647
Host species (*Q. rugosa*)	0.9169	0.6554	1.399	0.16183
Year 2 × *Q. rugosa*	0.0587	0.7462	0.079	0.93730
Year 3 × *Q. rugosa*	−20.3430	7179.93	−0.003	0.99774
Capsules produced per reproductive plant at time t*				
Intercept	−0.7825	0.4918	−1.591	0.111585
Size at start	0.4162	0.1160	3.590	0.000331
Host species (*Q. rugosa*)	0.5830	0.3702	1.575	0.115327

[†]Binomial (logit) GLMM.
[§]GLMM with Gaussian error structure and an exponential variance structure.
*Negative binomial GLMM.

Oncidium brachyandrum

Survival

For plants on both host species, survival increased as a function of plant size. There was no difference in survival between host species during the wettest year, but survival on the semi-deciduous *Q. rugosa* was higher than on the deciduous *Q. martinezii* during the dry and intermediate years (Table 4 and Figure 3A). The probability of mortality due to desiccation decreased significantly with (log) size ($\beta = -0.64$, SE = 1.07, $z = -6.00$, $p < 0.001$) and was significantly higher in the intermediate year than in the wettest year ($\beta = -1.63$, SE = 0.57, $z = -2.89$, $p = 0.004$). It did not differ significantly between host trees.

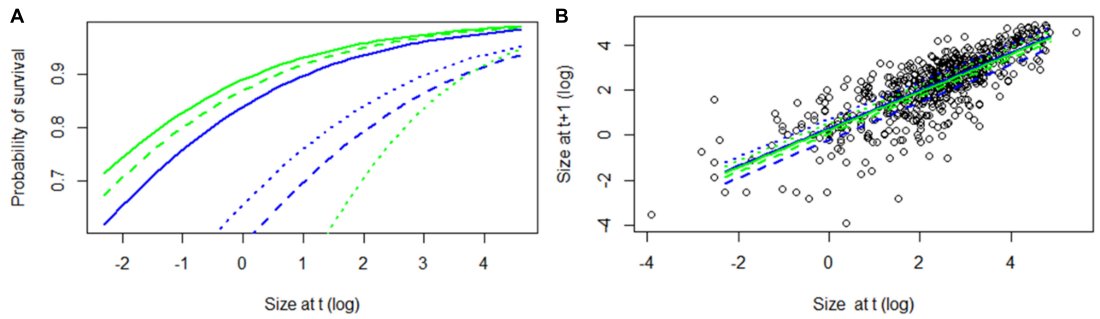

FIGURE 2
(A) Survival and **(B)** growth as function of size, for *Alamania punicea* plants growing on two oak species *Quercus martinezii* (blue) and *Q. rugosa* (green). Solid lines indicate year 2017–2018, dashed lines year 2018–2019, and dotted lines year 2019–2020.

TABLE 4 Estimated coefficients from mixed-effect models of the probability of survival, growth, reproduction, and probability of producing capsules for *Oncidium brachyandrum* plants growing on two *Quercus* species.

Fixed effects	Estimate	SE	*Z* value	*P*-value
Probability of surviving to $t+1$[†]				
Intercept	2.021234	0.363086	5.567	$2.59e-08$
Size at start	0.404343	0.078653	5.141	$2.74e-07$
Year 2 (2018–2019)	−0.500072	0.275850	−1.813	0.0699
Year 3 (2019–2020)	−0.357534	0.331662	−1.078	0.2810
Host species (*Q. rugosa*)	−0.009813	0.598163	−0.016	0.9869
Year 2 × *Q. rugosa*	1.320310	0.661457	1.996	0.0459
Year 3 × *Q. rugosa*	2.523453	1.119749	2.254	0.0242
Size at $t+1$ of surviving individuals (growth)[§]				
Intercept	−0.27854	0.05545	−5.02	$6.08e-07$
Size at start	0.74661	0.02133	35.01	< 2e-16
Year 2 (2018–2019)	0.26731	0.08206	3.26	0.00112
Year 3 (2019–2020)	0.82965	0.08549	9.70	< 2e-16
Host species (*Q. rugosa*)	−0.06476	0.11067	−0.59	0.55847
Year 2 × *Q. rugosa*	0.42201	0.159992	2.64	0.00832
Year 3 × *Q. rugosa*	0.45812	0.16220	−2.82	0.00474
Probability of producing capsules at time t (for individuals ≥ 35 cm)[†]				
Intercept	−3.4918	0.4120	−8.476	< 2e-16
Size at start	1.5336	0.2978	5.150	$2.62e-07$
Host species (*Q. rugosa*)	−0.8107	0.4413	−1.837	0.0662
Capsules produce per reproductive plant at time t*				
Intercept	−0.02442	0.27662	−0.088	0.9297
Size at start	0.36621	0.19401	1.888	0.0591

[†]Binomial (logit) GLMM.
[§]GLMM with Gaussian error structure and an exponential variance structure.
*Negative binomial GLMM.

Growth

For plants growing on both host species, growth was higher in dry and intermediate years than in wettest year. Growth was higher on the semi-deciduous *Q. rugosa* than on the deciduous *Q. martinezii* only during the dry year (Table 4 and Figure 3B).

Reproduction

Plants began to reproduce once they reached 0.78 cm^2 (pseudobulb area); 14% of plants flowered and 100% of these produced capsules. The probability of reproduction and the number of capsules produced per reproductive plant both increased as a function of size (Table 4). Host species was included in the best fit model for the probability of reproduction, with reproduction higher on the deciduous *Q. martinezii* than the semideciduous *Q. rugosa* (Table 4).

Host tree effect on population dynamics

Population growth rates of *A. punicea* on the two host species were similar in the wettest year and the intermediate years (Δ λ between hosts = 0.01). However, population growth rates were significantly higher on the semideciduous host than the deciduous host during the driest year ((Δ λ = 0.28; Figure 4A). For *O. brachyandrum*, λ was slightly lower on *Q. martinezii* than on *Q. rugosa* during the wettest year (Δ λ = 0.03). However, the reverse was true during the driest and intermediate years, where λ values were significantly higher on *Q. rugosa* than on *Q. martinezii* (Δ λ = 0.15 and 0.13, respectively) (Figure 4B). For *O. brachyandrum*, λ values were higher in the dry and intermediate years, than in the wettest year.

LTRE analyses

For *A. punicea*, the higher λ value observed for populations on *Q. rugosa* in the driest year was mainly due to higher survival on that host tree. For the wettest and the intermediate rainfall year, there was little differences in λ values across hosts, but differences were due to higher survival on *Q. rugosa* during the wettest year growth, and higher growth on *Q. martinezii* during the intermediate year (Figures 5A–C). For *O. brachyandrum* higher growth and reproduction contributed the most to the higher λ value of *Q. martinezii* in wettest year. The higher λ

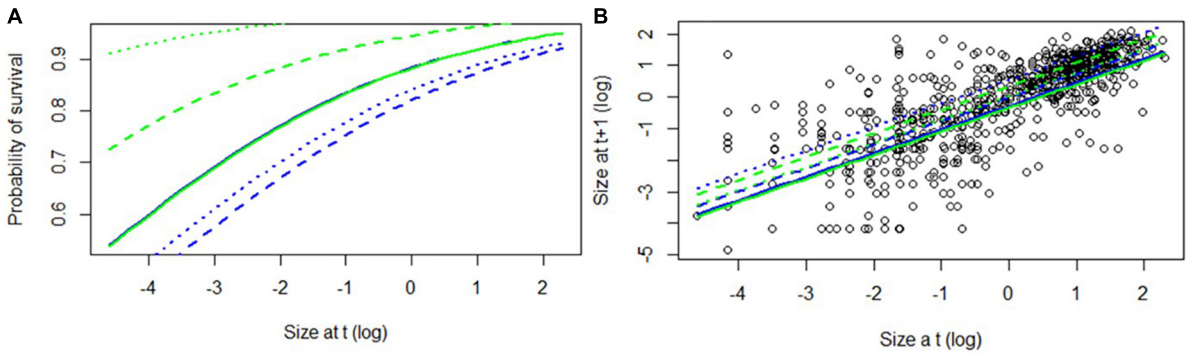

FIGURE 3
(A) Survival and **(B)** growth as function of size, for *Oncidium brachyandrum* plants growing on two oak species *Quercus martinezii* (blue) and *Q. rugosa* (green). Solid lines indicate year 2017–2018, dashed lines year 2018–2019, and dotted lines year 2019–2020.

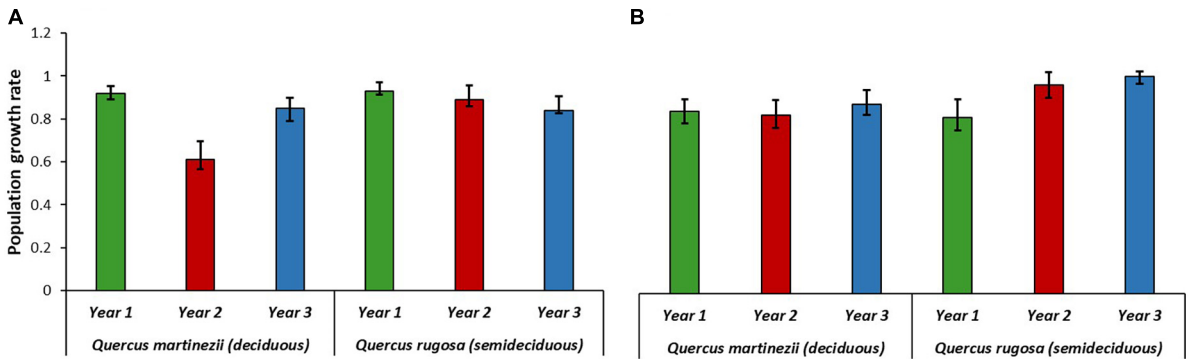

FIGURE 4
Population growth rates (λ values) of **(A)** *Alamania punicea* and **(B)** *Oncidium brachyandrum* growing on two *Quercus* species, from 2018 to 2020 in an oak forest in Oaxaca, Mexico. Error bars represent 95% confidence intervals.

values observed for populations in *Q. rugosa* during the two drier years was due to higher growth (driest year), and higher survival (intermediate year) (**Figures 5D–F**).

Discussion

The aim of our paper was to test if and how host species can affect the population dynamics of epiphytic orchids. Our results show that rates of survival, growth and reproduction of epiphytic orchids can vary across host tree species, and this can translate into difference in population growth rates. They also suggest that the direction and magnitude of these differences appears to depend on climatic conditions.

Our finding that both orchid species had higher growth and survival on the semi-deciduous host than on the deciduous host, during the driest year, but not during the wettest year, is consistent with other studies. Einzmann et al. (2015) found higher growth and survival rates of two epiphytic species growing on perennial trees than on deciduous trees, due to the sunnier and drier microclimates during dry season on the latter, which increased mortality due to desiccation. The same has been reported for an epiphytic bromeliad, where individuals growing on perennial pines had higher survival and growth rates that those growing on deciduous oak (Ticktin et al., 2016). Similarly, Callaway et al. (2002) reported higher growth rates of epiphytic individuals growing on their preferred host and attributed it to the higher water holding capacity its bark. Species with greater water holding capacity provide a more humid environment, and allow epiphytes access to water for a longer period. Humidity is recognized as the more limiting factor within epiphytism (Benzing, 1990; Laube and Zotz, 2003; Zotz, 2013).

Our results suggest potential lag effects of drought, since for the semi-deciduous host, a higher number of orchid individuals died the year after the driest year (intermediate year), than during the driest year itself. According to Zotz and Tyree (1996) there can be long-term drought stress effects on the physiology of orchids that can be perceived afterward forcing plants to show die back on some of their parts (like leaves), and finally die.

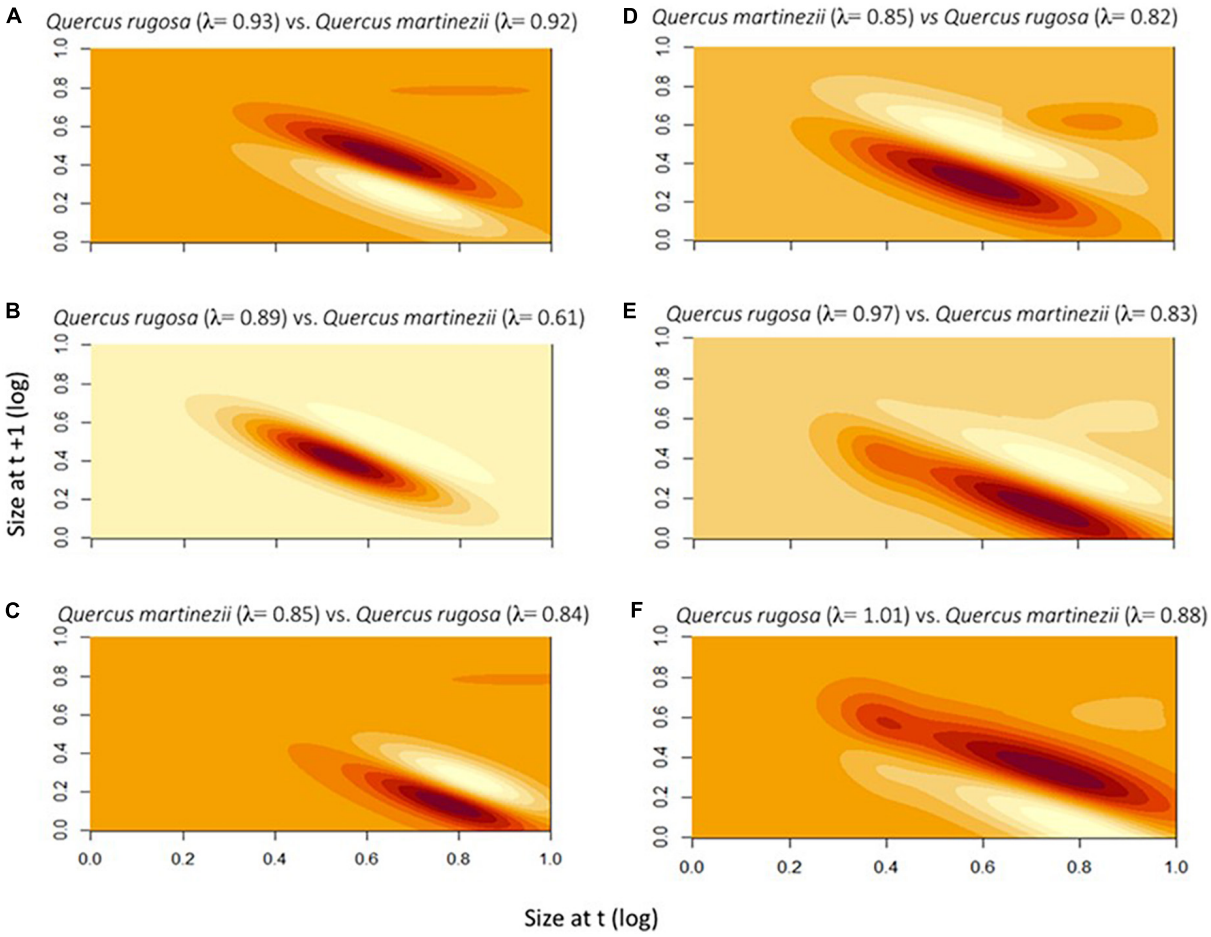

FIGURE 5
Life table response experiment contributions for *Alamania punicea* **(A–C)** and *Oncidium brachyandrum* **(D–F)** plants growing on two *Quercus* species. Darker colors represent life-history transitions that make greater contributions to higher λ values observed. Values across the diagonal represent contributions from survival, and those below diagonal represent contributions from growth. Fecundity contributions are represented in the top right corner.

Our finding that fecundity of *O. brachyandrum* was also higher on the deciduous host could be related to the low photosynthetic efficiency of epiphytic orchids due to drought adaptation (Sahagun-Godinez, 1996); they therefore need higher light (as in deciduous trees in our case) to perform photosynthesis efficiently and produce photosynthates for flower production. The lack of effect that we observed for *A. punicea* could be related to sample size, since very few individuals flowered during our study. The low probability of flowering is typical for many epiphytic orchids (Tremblay, 2006).

The differences we found in vital rates scaled up to differences in population growth rates, with λ values much lower on the deciduous host during the dry years than in the wettest year. Our finding that Δ λ between the wettest and dry year was greater for *A. punicea* than for *O. brachyandrum* is likely related to differences between the species in adaptation related with drought tolerance. *A. punicea* has thick leaves and cuticles, and small pseudobulbs (7–10 mm long), while *O. brachyandrum* has thin leaves and pseudobulbs 2–3 cm long. These represent two of the main strategies of orchids for drought tolerance (Stancato et al., 2001; Yang et al., 2016): thick cuticles avoid water loss, while pseudobulbs store water. In our study, the species with pseudobulbs better buffered the effect of host tree on its growth and survival rates. Pseudobulbs play an important role in the growth and survival of epiphytic orchids since they not only store water but are also responsible for the partition of assimilates, carbohydrate and minerals and can perform photosynthesis (Ng and Hew, 2000). Further research is needed to identify how morphological and physiological variation in epiphytic orchids (Dressler, 1993; Yang et al., 2016; Zhang et al., 2018) shapes demographic responses. In addition, translocation experiments could further disentangle differences in demographic rates across hosts.

Our results highlight the importance of preserving tree species diversity to foster the long-term persistence of populations of vascular epiphytes. This is especially true if we consider that vascular epiphytes function as metapopulations (Winkler et al., 2009; Valverde and Bernal, 2010), where individuals growing on one host tree represent a sub-population interconnected with other sub-populations (on other host trees) by seed dispersal, and where trees that support growing sub-populations determine the growth of the metapopulation (Winkler et al., 2009). Thus, metapopulation growth may be maximized when there is a diversity of host species that allow for growth under varying climatic conditions. In addition, variation among genotypes of the same tree species could potentially affect demographic rates, given that Zytynska et al. (2011) found a positive correlation between the genetic distances among host trees and similarity among the community vascular epiphytes growing on them. Further research is needed to better understand how host tree traits shape the persistence of populations of epiphytic orchids. On the ground, working with local communities to help identify land-use options that maintain tree diversity will be key.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

AR-M conducted fieldwork and wrote most of the manuscript. DM conceived the study, provided the advice, financial support, conducted fieldwork, and contributed to the manuscript. TT provided the advice, helped with data analyses, contributed to the manuscript, and reviewed the English. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Will Greenland be the last refuge for the continental European small-white orchid?Niche modeling of future distribution of *Pseudorchis albida*

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Climate change affects populations of plants, animals, and fungi not only by direct modifications of their climatic niches but also by altering their ecological interactions. In this study, the future distribution of suitable habitats for the small-white orchid (*Pseudorchis albida*) was predicted using ecological niche modeling. In addition, the effect of global warming on the spatial distribution and availability of the pollen vectors of this species was evaluated. Due to the inconsistency in the taxonomic concepts of *Pseudorchis albida*, the differences in the climatic preferences of three proposed subspecies were investigated. Due to the overlap of both morphological and ecological characters of ssp. *albida* and ssp. *tricuspis*, they are considered to be synonyms, and the final analyses were carried out using ssp. *albida* s.l. and ssp. *straminea*. All of the models predict that with global warming, the number of suitable niches for these orchids will increase. This significant increase in preferred habitats is expected to occur in Greenland, but habitat loss in continental Europe will be severe. Within continental Europe, *Pseudorchis albida* ssp. *albida* will lose 44%–98% of its suitable niches and *P. albida* ssp. *straminea* will lose 46%–91% of its currently available habitats. An opposite effect of global warming was predicted for pollinators of *P. albida* s.l., and almost all insects studied will be subject to habitat loss. Still, within the predicted potential geographical ranges of the orchid studied, some pollen vectors are expected to occur, and these can support the long-term survival of the small-white orchid.

KEYWORDS

bioclimatic preferences, habitat loss, global warming, pollinators, *Pseudorchis albida* ssp. *tricuspis*, *Pseudorchis albida* ssp. *straminea*

1 Introduction

The sole representative of the genus *Pseudorchis* Ség., the small-white orchid (*Pseudorchis albida* (L.) Á. Löve and D. Löve), is a tuberous perennial geophyte growing in most of Europe and northern Asia from Spain and Iceland to northwest Siberia (Jersáková et al., 2011). This species is variable within its geographical range, and the morphological differences prompted taxonomists to divide *P. albida* into three subspecies: ssp. *albida,* ssp. *straminea* (Fern.) Ä. Löve and D. Löve, and ssp. *tricuspis* (Beck) Klein (Figure 1; Reinhammar, 1998; Klein, 2000; Jersáková et al., 2011). However, the recognition of these taxa is still debated. Reinhammar (1995, 1998) recognized two species in the genus *Pseudorchis*, with moderate morphometric support. The studies on their allozymes also indicate that it is reasonable to accept the species status of the lowland to subalpine *P. albida* s.s., and alpine *P. straminea* (Reinhammar and Hedren, 1998). Klein (2000) accepted three subspecies of *P. albida*: ssp. *tricuspis* (calcicolous, with an alpine–boreal distribution), ssp. *albida* (acidophilous, with alpine–temperate–boreal distribution), and ssp. *straminea* (basiphilous, with west Arctic–north Atlantic distribution). Jersáková et al. (2011) considered that the taxa of *Pseudorchis* characterized by differences in distribution are not well-defined and accept the broad concept of *P. albida* s.l. On the other hand, Bateman et al. (2017) recognized *Pseudorchis albida* and *P. straminea* as separate species, pointing out morphological features and molecular divergence (ITS) sufficient for species-level distinction. The same authors rejected the separateness of *P. tricuspis* due to overlap in supposedly taxonomically useful characters with *P. albida* and *P. straminea* (Bateman et al., 2017). Considering the differences in the taxonomic approach, it was decided to accept all taxa as subspecies in this ecological study. The results of the analyses can be used in further taxonomic studies on *Pseudorchis.*

Pseudorchis albida ssp. *albida* occurs in areas with a boreal–montane climate and is found from United Kingdom across Scandinavia to the northern Urals in the European part of Russia as well as in mountain ranges from Spain across the Alps to the Eastern Carpathians (Jersáková et al., 2011). *Pseudorchis albida* ssp. *straminea* is restricted to areas with a west Arctic–north Atlantic climate (Iceland, Faroes, Greenland, and Scandinavia; Jersáková et al., 2011), and *Pseudorchis albida* ssp. *tricuspis* is restricted to alpine–boreal areas (Swiss, Italian and Austrian Alps, Tatra Mountains, and Eastern Carpathian; Jersáková et al., 2011).

Populations of small-white orchid reproduce mainly sexually (Jersáková et al., 2011), and vegetative propagation by tubers contributes little to population growth (Summerhayes, 1951). As a species that provides a nectar reward, several species of Lepidoptera (Claessens and Kleynen, 2011) are reported pollinators of *Pseudorchis albida.* More recently, Jersáková et al. (2011) also reported species of *Empis* (Diptera) as diurnal pollen vectors.

In terms of anthropogenic threats, *P. albida* is endangered by agricultural development and afforestation (Reinhammar et al., 2002; Foley and Clarke, 2005; Forbes and Northridge, 2012). Reduction in traditional mowing and grazing has resulted in it being overgrown by more competitive species (Reinhammar et al., 2002; Holland et al., 2008). On the other hand, reduced seed set and recruitment can result from over-grazing (Duffy et al., 2009; Jersáková et al., 2011). The effect of global warming on this species is yet to be evaluated.

According to the IUCN Red List, *Pseudorchis albida* is assessed as a species of least concern because it is rather widespread (Rankou, 2011). However, due to a considerable decline in its distribution, it is currently considered to be critically endangered in Greece (small population found by Tsiftsis and Antonopoulos, 2011), vulnerable in Great Britain (Cheffings and Farrell, 2005) and Bulgaria (Petrova and Vladimirov, 2009), endangered in Ireland (Curtis and McGough, 1988), Czech Republic (Holub and Procházka, 2000), Germany (Ludwig and Schnittler, 1996), and Sweden (Gärdenfors, 2010), and near threatened in Norway (Artsdatabanken, 2010) and Poland (Kaźmierczakowa et al., 2016). It is also protected in many European countries (Reinhammar et al., 2002; Bilz et al., 2011), e.g., Poland (Kaźmierczakowa et al., 2016), Czech Republic (Danihelka et al., 2012), Denmark (Damgaard et al., 2020), Romania (Sârbu et al., 2020), Ukraine (Kricsfalusy et al., 1999, 2010), Slovakia (Turis et al., 2014), Norway (subordinate agency, 2022), Sweden (Naturva°rdsverket, 2022), Austria (Zulka et al., 2001; Jersáková et al., 2011), Germany (Jersáková et al., 2011), Switzerland (Jersáková et al., 2011), and Italy (Jersáková et al., 2011).

This study aimed to estimate the effect of global warming on the distribution of climatic niches suitable for *P. albida* s.l. Since this orchid relies mainly on sexual reproduction, the effect of climate change was also evaluated for the pollinators of this orchid. To improve the estimates and because the taxonomic separateness of *P. albida* ssp. *tricuspis* is questioned by some authors (Bateman et al., 2017), the differences in the preferred climatic niches of the three-known subspecies of *P. albida* were evaluated in order to assess their ecological distinctiveness.

2 Materials and methods

2.1 List of localities

The databases of localities of *Pseudorchis albida* s.l. in continental Europe as well as records of pollinators of this orchid were compiled based on information in public facilities accessed through the Global Biodiversity Information Facility (GBIF 2020; Supplementary Table S1). The information on pollen vectors was obtained from previous reports on pollination of *P. albida* by Claessens and Kleynen (2011) and Jersáková et al. (2011). There was an insufficient number of occurrences for *Empis bistortae* Meigen, 1822 for performing an

FIGURE 1
Photographs of the small-white orchid in its natural habitat. *Pseudorchis albida* ssp. *albida* in Rhön, Germany **(A),** and Zillertal Alps, Austria [**(B)**; photographer: Marco Klüber/www.m-klueber.de], *Pseudorchis albida* ssp. *straminea* in Newfoundland, Canada [**(C,D)**; photographer: James Fowler], and *Pseudorchis albida* ssp. *tricuspis* on Mt. Mangart, Julian Alps, Slovenia [**(E,F)**; photographer: Amadej Trnkoczy].

analysis for this insect. From a total of 4518 localities for *Pseudorchis albida* (ssp. *albida*—316, ssp. *straminea*—4170, and *tricuspis*—32) and 69424 for insects (*Chrysoteuchia culmella* (Linnaeus, 1758)—46299, *Crambus ericella* (Hübner, 1813)—1098, *Crambus pascuella* L.—4032, *Plutella xylostella* (Linnaeus, 1758)—17643, and *Udea uliginosalis* (Stephens, 1834)—352) available in the repositories, only records that were georeferenced with a minimum of 1 km precision were selected. To reduce sampling bias, spatial thinning was carried out using SDMtoolbox 2.3 for ArcGIS (Kremen et al., 2008; Brown, 2014). The data were rarified by designating a minimal distance of 5 km for calculating climatic habitat heterogeneity. The final database included 28 localities for *P. albida* ssp. *albida*, 414 for *P. albida* ssp. *straminea*, 11 for *P. albida* ssp. *tricuspis* (Supplementary Data Sheet S1), and 3694 for its pollinators (*Chrysoteuchia culmella*—1472, *Crambus ericella*—249, *Crambus pascuella*—707, *Plutella xylostella*—1244, and *Udea uliginosalis*—22; Supplementary Data Sheet S2).

2.2 Principal component analysis

Principal components analysis (PCA) was used to evaluate the differences between populations of *P. albida* ssp. *straminea*, *P. albida* ssp. *albida*, and *P. albida* ssp. *tricuspis* based on 19 bioclimatic variables from WorldClim v. 2.1 (Table 1; Fick and Hijmans, 2017). Calculations were carried out using the software package Statistica PL. ver. 13.3 (StatSoft Inc. 2011). The data matrix was transformed (square root) before carrying out the ordination analysis.

2.3 Ecological niche modeling

The modeling of the current and future distribution of the species studied was carried out using the maximum entropy method implemented in MaxEnt version 3.3.2 (Phillips et al., 2004, 2006; Elith et al., 2011) based on presence-only

TABLE 1 List of variables used in the PCA and modeling (with an asterisk).

Variable code	Description
bio1*	Annual mean temperature
bio2*	Mean diurnal range (mean of monthly (max temp–min temp)
bio3*	Isothermality (bio2/bio7) (×100)
bio4*	Temperature seasonality (standard deviation ×100)
bio5	Max temperature in the warmest month
bio6	Min temperature in the coldest month
bio7	Temperature annual range (bio5–bio6)
bio8*	Mean temperature in the wettest quarter
bio9*	Mean temperature in the driest quarter
bio10	Mean temperature in the warmest quarter
bio11	Mean temperature in the coldest quarter
bio12*	Annual precipitation
bio13	Precipitation in the wettest month
bio14*	Precipitation in the driest month
bio15*	Precipitation seasonality (coefficient of variation)
bio16	Precipitation in the wettest quarter
bio17	Precipitation in the driest quarter
bio18*	Precipitation in the warmest quarter
bio19	Precipitation in the coldest quarter

observations. For the modeling, bioclimatic variables in 30 arc-seconds of the interpolated climate surface downloaded from WorldClim v. 2.1 were used (Fick and Hijmans, 2017). Nine of 19 variables were removed from the analyses due to their high correlation with other variables as indicated by Pearson's correlation coefficient (Table 1; Supplementary Data Sheet S3) computed using SDMtoolbox 2.3 for ArcGIS (Kremen et al., 2008; Brown, 2014). Because some previous studies (Barve et al., 2011) suggest that modeling based on data for a restricted area is more reliable than calculating habitat suitability at a global scale, the area included in the analysis was restricted to 84.65–34.43°N and 74.65°W–45.43°E. Since this study investigated the effect of climate change on the distribution of the species and soil characteristics have little effect on models of Australian terrestrial orchid, *Leporella fimbriata* (Kolanowska et al., 2021a), we did not use these variables in the analyses.

Predictions of the future extent of the climatic niches of *P. albida* and its pollinator in 2080–2100 were made using climate projections developed by the CNRM/CERFACS modeling group for the coupled model intercomparison project (CNRM–CM6-1) for four shared socio-economic pathways (SSPs; O'Neill et al., 2014): SSP1-2.6, SSP2-4.5, SSP3-7.0, and SSP5-8.5. The layers in 2.5 arc-minutes were re-scaled to fit bioclimatic variables. SSPs are trajectories adopted by the Intergovernmental Panel on Climate Change (IPCC), which provide a broader view of a "business as usual" world without a climate policy, with global warming in 2100 ranging from a low of 3.1°C to a high of 5.1°C above pre-industrial levels (O'Neill et al., 2014).

In all the analyses, the maximum number of iterations was set to 10000 and that of convergence threshold to 0.00001. The neutral (= 1) regularization multiplier value and auto features were used. All samples were added to the background. The "random seed" option, which provided a random test partition and background subset for each run, was applied, and 20% of the samples were used as test points. The run was performed as a bootstrap with 100 replicates. The output was set to logistic. In addition, the "fade by clamping" function in MaxEnt was enabled to preclude extrapolations outside the environmental range of the training data (Phillips et al., 2006). All analyses of GIS data were carried out on ArcGIS 10.6 (Esri, Redlands, CA, United States). The evaluation of the models was conducted using the area under the curve (AUC; Mason and Graham 2002; Evangelista et al., 2008) and True Skill Statistic (TSS; Allouche et al., 2006).

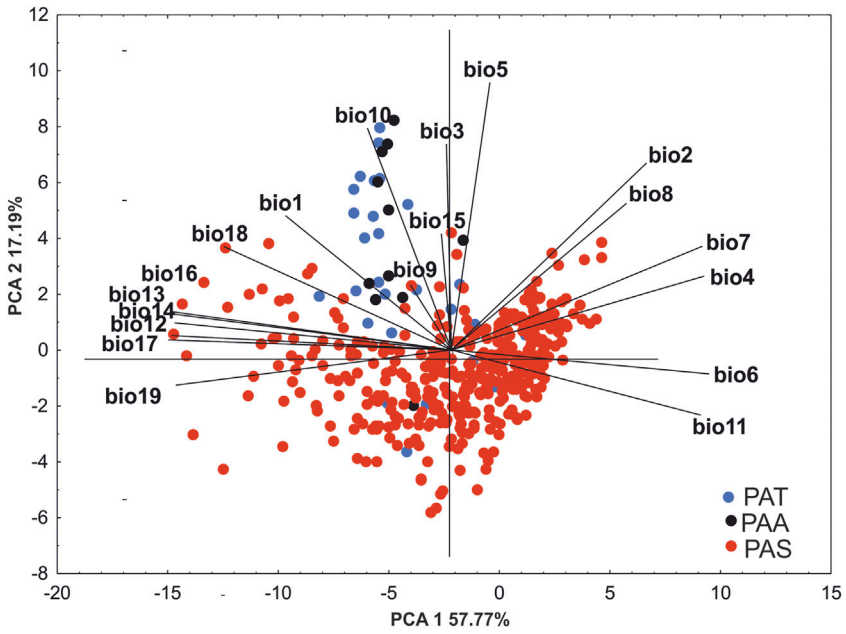

FIGURE 2
PCA ordination diagram (principal component analysis) of the distributions of populations of *P. albida* ssp. *straminea* (red dots), *P. albida* ssp. *albida* (black dots), and *P. albida* ssp. *tricuspis* (blue dots) based on 19 bioclimatic variables.

SDMtoolbox 2.3 for ArcGIS (Kremen et al., 2008; Brown, 2014) was used to visualize changes in the distribution of suitable niches of the orchid studied and its pollinator due to global warming. To compare the prediction of the model of the current distribution with future predictions, all SDMs were converted into binary rasters and projected using the Goode homolosine. The presence threshold was estimated based on the values for grids in which the species studied were predicted to occur using present-time data. Because about 70%–84% of known localities of *P. albida* and its pollinators were located in grids with values > 0.4, this threshold value was used to create binary rasters. To determine the availability of pollinators for the orchid, the overlap of the binary models of both organisms was calculated.

3 Results

3.1 Ecological differences between subspecies of *Pseudorchis*

The result of PCA analyses indicate that although the preferred niche of *P. albida* ssp. *straminea* differs from that of the two other taxa, *P. albida* ssp. *albida* and *P. albida* ssp. *tricuspis* occupy similar habitats. This is indicated by the second axis, which separated *P. albida* ssp. *albida* and *P. albida* ssp. *tricuspis* from most of the records of *P. albida* ssp. *straminea*. Our analyses indicate significant differences in the bioclimatic preferences of the subspecies. of *P. albida*. Along the gradient represented by the first axis, *P. albida* ssp. *straminea* is correlated especially with precipitation in the warmest quarter (bio18) and the mean temperature in the wettest quarter (bio8). The ordination diagrams of PCA explained 68.96% of the total variance. The first component accounted for 51.77% of the total variance and the second for 17.19% (Figure 2; Supplementary Table S2). Based on the morphological similarities of the two latter orchids, a broader concept of *P. albida* ssp. *albida* was used, which also includes *P. albida* ssp. *tricuspis*.

3.2 Model evaluation and limiting factors

The models had high AUC (0.871–0.998) and TSS (0.517–0.9924) scores, indicating their predictions are very reliable (Figure 3; Table 2). The most important variable limiting the distribution of *P. albida* ssp. *albida* was precipitation in the warmest quarter (bio18—47.5%). Much less significant for its occurrence were the annual precipitation (bio12—18.5%) and the annual mean temperature (bio1—14.9%). The latter factor was crucial (42.6%) for the distribution of *P. albida* ssp. *straminea*, followed by the mean temperature in the wettest quarter (bio8—29.8%) and precipitation in the warmest quarter (bio18—7.9%).

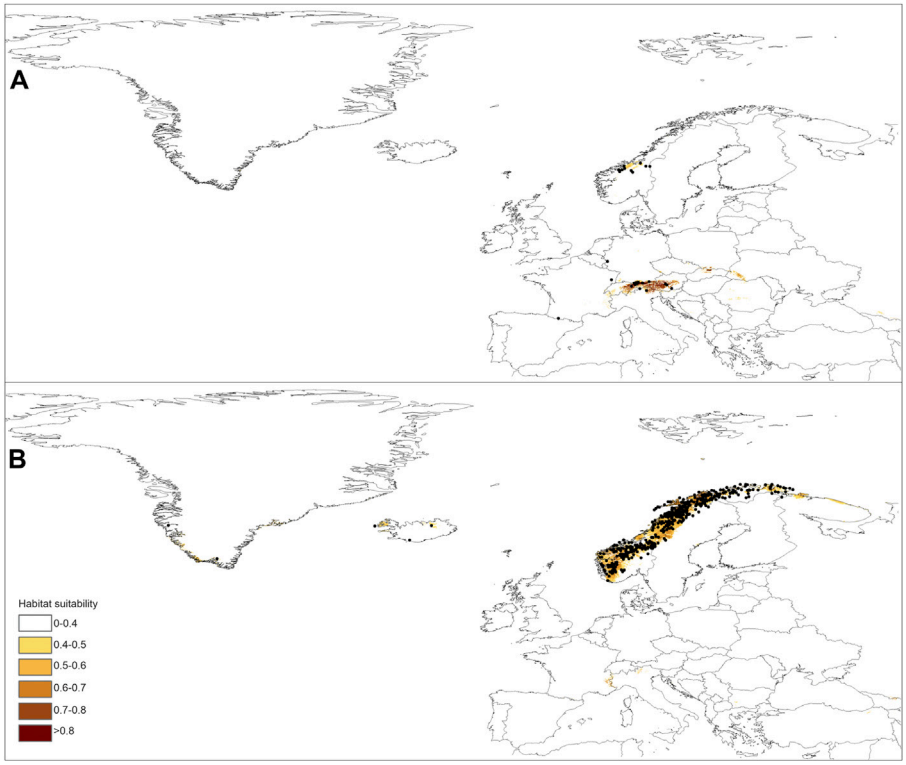

FIGURE 3
Current distribution of suitable niches for *P. albida* ssp. *albida* **(A)** and *P. albida* ssp. *straminea* **(B)** along with the localities included in the models (marked by black dots).

3.3 Effect of climate change on *P. albida* and its pollinators

The predictions of the present-time models are congruent with the known geographical ranges of *P. albida* ssp. *albida* and *P. albida* ssp. *straminea* (Figure 3). Our analyses indicate the critical changes in the distribution of small-white orchid (Figures 4–7). All models predict that the availability of suitable niches for the orchids studied will increase as a result of global warming (Table 3), but the significant increase in suitable niches is expected to occur in Greenland, whereas habitat loss in continental Europe will be severe. Overall, the potential range of *P. albida* ssp. *albida* will be 27%–88% greater than at present, whereas that of *P. albida* ssp. *straminea* will be 88%–156% greater. The unexpected result is that while SSP1-2.6 is expected to be the most advantageous climate change scenario for the latter taxon, the same scenario is the least optimistic for *P. albida* ssp. *albida*, which will mostly benefit from SSP5-8.5.

Pseudorchis albida ssp. *albida* is currently known to occur only in continental Europe, but apparently its suitable habitats will be located mainly in Greenland in the future and will become extinct in continental Europe based on SSP5-8.5. *P. albida* ssp. *straminea* will also face significant loss of habitats in this part of its range; however, it could potentially extend its range to Svalbard (only in the less severe scenarios, such as SSP1-2.6 and SSP2-4.5, is its occurrence in Iceland not completely threatened). Within continental Europe, *Pseudorchis albida* ssp. *albida* will lose 44% (SSP1-2.6)–99% (SSP5-8.5) of its suitable niches, and *P. albida* ssp. *straminea* will lose 46% (SSP1-2.6)–91% (SSP5-8.5) of its current habitat.

While in the future *P. albida* is predicted to occupy different areas, the situation is completely different for the pollinators of this species (Table 3). All models predict a significant loss of habitat for them, which in the case of *Udea uliginosalis* could result in its extinction (Table 3).

3.4 Availability of pollinators

Based on the analyses, *Udea uliginosalis* is currently present in ca. 10% of the potential range of *P. albida* ssp. *straminea*, but will not be present there by 2100 (Supplementary Data Sheet S4; Table 4).

Plutella xylostella is predicted to be the most important pollinator of *P. albida*, with a range overlap of 75% (SSP5-8.5)–88% (SSP3-7.0) with *P. albida* ssp. *albida* and 70% (SSP2-4.5)–100% (SSP1-2.6) with *P. albida* ssp. *straminea*. *Chrysoteuchia*

TABLE 2 TSS scores, average training AUC, and standard deviations (in brackets) for the replicate runs of the models.

Species	Scenario	TSS	AUC
P. albida ssp. *albida*	Present	0.9388	0.994 (0.001)
	SSP1-2.6	0.9553	0.995 (0.001)
	SSP2-4.5	0.9410	0.994 (0.001)
	SSP3-7.0	0.9124	0.994 (0.001)
	SSP5-8.5	0.9416	0.993 (0.001)
P. albida ssp. *straminea*	Present	0.9158	0.973 (0.001)
	SSP1-2.6	0.9172	0.973 (0.001)
	SSP2-4.5	0.9196	0.974 (0.001)
	SSP3-7.0	0.9217	0.973 (0.001)
	SSP5-8.5	0.9220	0.973 (0.001)
Chrysoteuchia culmella	Present	0.6317	0.884 (0.002)
	SSP1-2.6	0.6333	0.888 (0.002)
	SSP2-4.5	0.6412	0.884 (0.002)
	SSP3-7.0	0.6520	0.887 (0.002)
	SSP5-8.5	0.6341	0.885 (0.002)
Crambus ericella	Present	0.7740	0.957 (0.003)
	SSP1-2.6	0.7740	0.960 (0.003)
	SSP2-4.5	0.7740	0.957 (0.003)
	SSP3-7.0	0.7740	0.958 (0.004)
	SSP5-8.5	0.7740	0.958 (0.003)
Crambus pascuella	Present	0.7208	0.921 (0.003)
	SSP1-2.6	0.7112	0.922 (0.003)
	SSP2-4.5	0.6886	0.919 (0.002)
	SSP3-7.0	0.7096	0.922 (0.003)
	SSP5-8.5	0.7208	0.920 (0.002)
Plutella xylostella	Present	0.5356	0.872 (0.003)
	SSP1-2.6	0.5323	0.877 (0.003)
	SSP2-4.5	0.5281	0.871 (0.003)
	SSP3-7.0	0.5170	0.875 (0.003)
	SSP5-8.5	0.5271	0.872 (0.003)
Udea uliginosalis	Present	0.9800	0.997 (0.001)
	SSP1-2.6	0.9425	0.997 (0.001)
	SSP2-4.5	0.9843	0.997 (0.001)
	SSP3-7.0	0.9806	0.997 (0.001)
	SSP5-8.5	0.9924	0.998 (0.001)

culmella is currently present in 74% of the range of *P. albida* ssp. *albida* and 52% of that of *P. albida* ssp. *straminea*. The predicted future distribution of this insect will overlap partially with both subspecies of the small-white orchid, overlapping 66% (SSP1-2.6)–91% (SSP3-7.0) of that of *P. albida* ssp. *albida* and 38% (SSP2-4.5)–56% (SSP3-7.0) of that of *P. albida* ssp. *straminea*. The statistics for *Crambus ericella* and *C. pascuella* are similar (Table 4).

4 Discussion

4.1 Implication for taxonomy

The recognition of three taxa within the *Pseudorchis albida* group remains a topic of taxonomic discussion and concern in terms of both their distinction and rank. This study indicates that ssp. *tricuspis* occupies niches similar to those occupied by ssp. *albida*, even if ssp. *tricuspis* is considered to be an alpine taxon and ssp. *albida* associated with lowland to subalpine regions (Reinhammar et al., 2002; Jersáková et al., 2011). On the other hand, Klein (2000) argues that ssp. *tricuspis* should be considered to be a separate subspecies, and this concept is also accepted by other scientists (Moore, 1980; Reinhammar, 1998; Bournérias and Prat, 2005; Perazza, 2016). Reinhammar (1995), Reinhammar (1998) based on the results of a multivariate morphometric study considering plants of ssp. *tricuspis* as conspecific with *P. straminea*. The position of "*tricuspis*" as a variety is proposed by Kreutz (2004), Delforge (2006), and Jersáková et al. (2011). Landwehr (1977) believes that this taxon is just a form of *P. albida*. Unfortunately, no molecular studies have included ssp. *tricuspis*. The results presented indicate that their morphological characteristics are very similar, which supports merging them under ssp. *albida*.

Unlike *Pseudorchis albida* ssp. *tricuspis*, ssp. *straminea* is more distinct. Only the rank of this taxon is debated. Analyses presented in this paper reveal differences in the climatic requirements of ssp. *albida* and ssp. *straminea,* which could be a potential argument and area for research on whether to elevate the latter taxon to a separate species. This is proposed based on its morphology (Reinhammar 1995; Reinhammar 1998) and differences in allozymes (Reinhammar and Hedren, 1998). According to Duffy et al. (2011) the AFLP markers for *P. albida* are very polymorphic, and there are significant differences both within and among populations, and population genetic isolation increases with distance but did not find any differences in plastid microsatellites between Irish populations of ssp. *albida* and Swedish ssp. *straminea*. Based on molecular studies, Bateman et al. (2003); Bateman et al. (2017) show that the differences in DNA sequences (nrITS, *rbcL*, and *trnL-F*) of the two taxa are near the lowest level of acceptance for their being separate species. Bateman et al. (2017) also reported at least 14 morphometric characters that can be used to identify these taxa. Based on

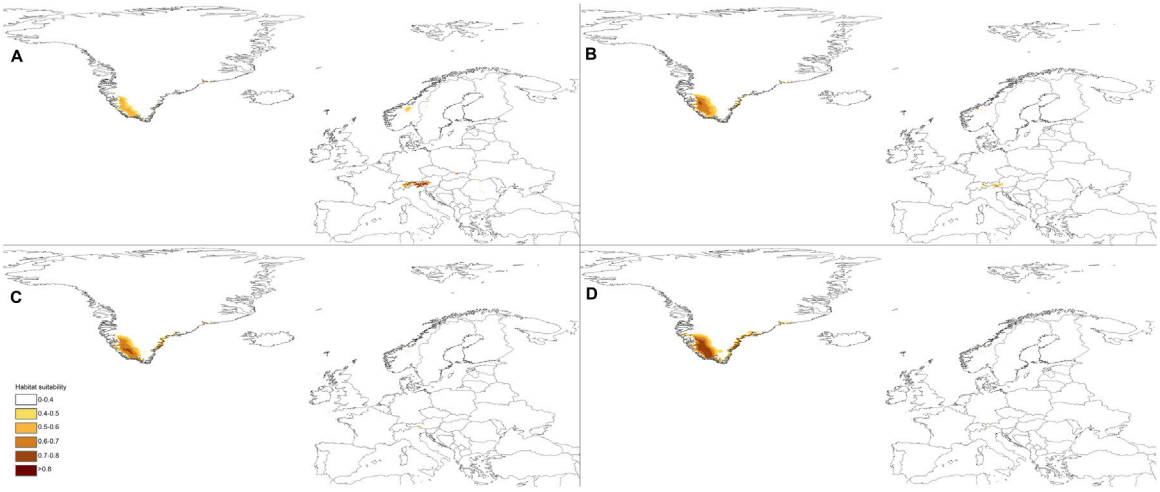

FIGURE 4
Future distribution of suitable niches for *P. albida* ssp. *albida* predicted under SSP1-2.6 **(A)**, SSP2-4.5 **(B)**, SSP3-7.0 **(C)**, and SSP5-8.5 **(D)** climates.

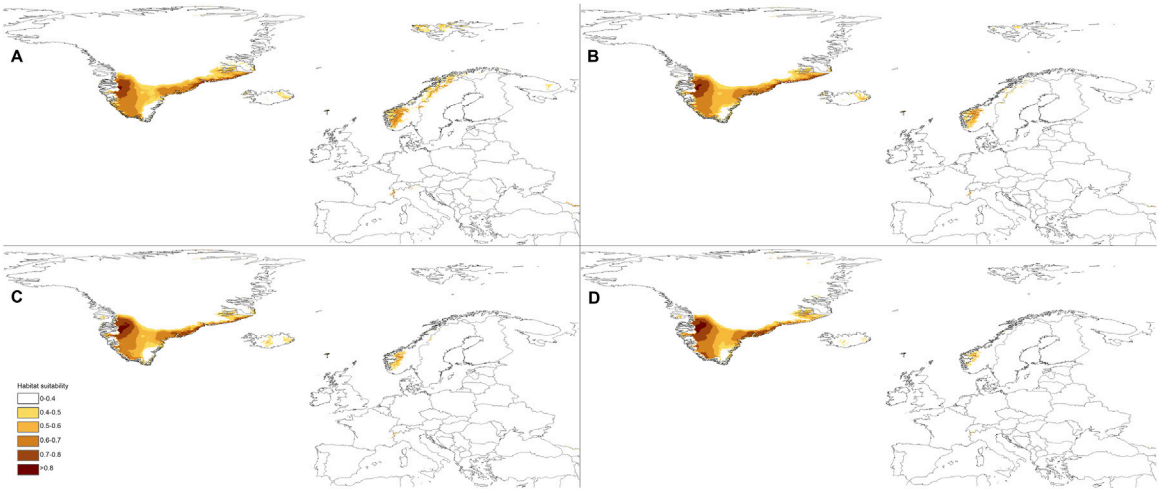

FIGURE 5
Future distribution of suitable niches for *P. albida* ssp. *straminea* predicted under SSP1-2.6 **(A)**, SSP2-4.5 **(B)**, SSP3-7.0 **(C)**, and SSP5-8.5 **(D)** climates.

previous studies and the results presented, it is proposed that "*straminea*" is a subspecies.

4.2 Effect of global warming on occurrence of *P. albida s.l.* and its conservation

The effect of predicted climate change will adversely affect populations of *P. albida* in continental Europe. In the best-case scenario (SSP1-2.6) both subspecies, ssp. *albida* and ssp. *straminea*, will lose almost half of their current suitable niches (44% and 46%, respectively). In the most damaging SSP5-8.5, only 1%–9% of the currently available habitats will still be suitable for small-white orchids. Global warming is one of the most important causes of changes in habitat (Opdam and Wascher, 2004; Troia et al., 2019). This is particularly so for alpine species, the available habitat for which is likely to significantly decrease (Freeman et al., 2018; Lamprecht et al., 2018) and other species with

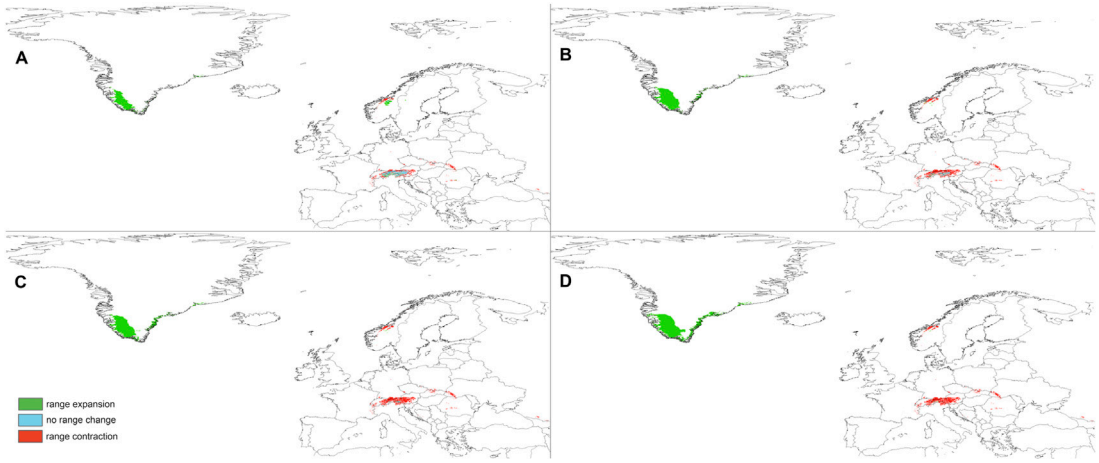

FIGURE 6
Changes in the distribution of suitable niches for *P. albida* ssp. *albida* predicted under SSP1-2.6 **(A)**, SSP2-4.5 **(B)**, SSP3-7.0 **(C)**, and SSP5-8.5 **(D)** climates.

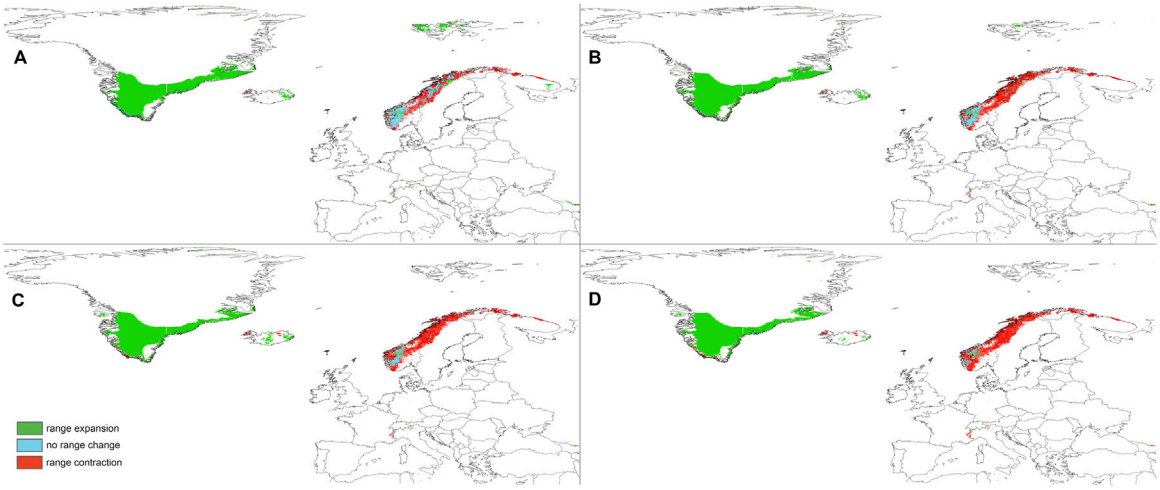

FIGURE 7
Changes in the distribution of suitable niches for *P. albida* ssp. *straminea* predicted under SSP1-2.6 **(A)**, SSP2-4.5 **(B)**, SSP3-7.0 **(C)**, and SSP5-8.5 **(D)** climates.

very specific ecological requirements (Tsiftsis et al., 2019). Geppert et al. (2020) indicated that ranges of some alpine orchids are or will decrease, especially since they are also threatened by other factors, i.e., habitat modification and loss of specific ecological relationships. Similar results are reported in a study on another orchid with a Scandinavian-alpine distribution in Europe, *Nigritella nigra s.l.* (Kolanowska et al., 2021b). However, global warming will result in the transformation of currently unsuitable habitats in Greenland. Shifts in the ranges of species may enable them to access and colonize these areas (Kelly and Goulden, 2008; Cannone and Pignatti, 2014; Geppert et al., 2020). However, as the populations of *P. albida* are usually very small (Jeřábková, 2006; Pearman et al., 2008; Jersáková et al., 2011), it is unlikely that ssp. *albida* will be able to colonize and adapt to new habitats in Greenland in the next few decades. These should be accessible for ssp. *Straminea,* which is more likely to be able to colonize this

TABLE 3 Changes in the coverage of suitable niches [km^2] for *P. albida* and its pollinators.

Species	Scenario	Range expansion	No change	Range contraction	Change
P. albida ssp. *albida*	SSP1-2.6	66505.58	31020.44	45720.92	+27.08%
	SSP2-4.5	96261.33	11635.83	65105.53	+40.60%
	SSP3-7.0	104480.4	1910.023	74831.34	+38.64%
	SSP5-8.5	144083.0	409.486	76331.88	+88.29%
P. albida ssp. *straminea*	SSP1-2.6	508313.6	104807.2	134675.1	+156.02%
	SSP2-4.5	485026.9	55570.46	183911.8	+125.74%
	SSP3-7.0	451762.1	30816.04	208666.3	+101.51%
	SSP5-8.5	436690.5	15737.52	223744.8	+88.92%
Chrysoteuchia culmella	SSP1-2.6	329513.2	1025813	575486.4	−15.36%
	SSP2-4.5	299677.4	903473	697826.3	−24.86%
	SSP3-7.0	312333.2	771202.9	830096.4	−32.33%
	SSP5-8.5	314846.2	616610.7	984688.7	−41.83%
Crambus ericella	SSP1-2.6	423770.1	331805.3	239292.9	+32.30%
	SSP2-4.5	362709.5	241766.3	329332	+5.84%
	SSP3-7.0	348517.7	139041.3	432056.9	−14.63%
	SSP5-8.5	247644.7	76063.9	495034.4	−43.32%
Crambus pascuella	SSP1-2.6	239193.1	808203.6	341319.9	−8.88%
	SSP2-4.5	345567.2	764014	385509.5	−3.47%
	SSP3-7.0	398812	678053.4	471470.1	−6.32%
	SSP5-8.5	442073.3	590718	558805.5	−10.15%
Plutella xylostella	SSP1-2.6	977527.1	1318081	586601.3	+20.52%
	SSP2-4.5	871332.8	1163715	740967.3	+6.84%
	SSP3-7.0	748247.1	1067649	837033.1	−4.66%
	SSP5-8.5	759111.1	1047278	857403.5	−5.16%
Udea uliginosalis	SSP1-2.6	286.4351	8737.296	18757.06	−67.18%
	SSP2-4.5	8.887008	1552.492	25941.86	−94.32%
	SSP3-7.0	4.101696	2.734464	27491.62	−99.98%
	SSP5-8.5	0	0	27494.35	−100.00%

area. That distributions of orchids can change as a result of global warming is unlikely, but is suggested in some previous studies (van der Meer et al., 2016; Kolanowska et al., 2017).

An important aspect of the occurrence of *Pseudorchis* in Greenland is that currently most of the island is covered by ice (GrIS). Studies indicate that by 2100, the thickness of the GrIS will decrease significantly, but the area occupied will not differ much (Muntjewerf et al., 2020; Greve and Chambers 2022; Yang et al., 2022). This means that many areas predicted suitable by the models will still be inaccessible to *Pseudorchis*, and its occurrence will be limited to the island's coastal zone. Of course, this has implications for the future, when the area of the GrIS is expected to decrease significantly and thus there will be new areas for colonization by plants (Chambers et al., 2022; Greve and Chambers 2022; Yang et al., 2022).

While a similar decline in the availability of a pollinator previously predicted for the Australian orchid *Leporella fimbriata* (Kolanowska et al., 2021a) is unlikely to affect *P. albida*, changes in climate will probably not limit the long-term survival of this species. According to data available in GBIF (Table 5), at the beginning of the flowering season (June–August) of both subspecies of *Pseudorchis*, their pollinators are active and

TABLE 4 Overlap of potential ranges of *P. albida* and its pollinators.

	Scenario	*C. culmella* (%)	*C. ericella* (%)	*C. pascuella* (%)	*P. xylostella* (%)	*U. uliginosalis* (%)
P. albida ssp. *albida*	Present	73.76	76.50	72.95	74.40	0.00
	SSP1-2.6	65.52	72.26	83.42	80.38	0.00
	SSP2-4.5	74.02	73.33	88.10	86.92	0.00
	SSP3-7.0	90.63	77.19	89.52	88.10	0.00
	SSP5-8.5	80.43	83.18	89.55	74.81	0.00
P. albida ssp. *straminea*	Present	51.91	57.94	57.34	46.11	10.98
	SSP1-2.6	44.55	61.87	47.92	100.00	0.00
	SSP2-4.5	37.60	55.49	39.39	69.60	0.00
	SSP3-7.0	56.03	64.79	48.07	74.74	0.00
	SSP5-8.5	52.12	67.04	59.89	72.34	0.00

TABLE 5 Overlap of potential ranges of *P. albida* and its pollinators.

Species	Month											
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
P. albida ssp. *albida*						x	x	x	x	x		
P. albida ssp. *straminea*						x	x	x	x	x		
Chrysoteuchia culmella						x	x	x	x			
Crambus ericella						x	x	x				
Crambus pascuella					x	x	x	x	x	x		
Plutella xylostella			x	x	x	x	x	x	x	x		
Udea uliginosalis						x	x	x				

can transfer pollen. For September and October, there are no reports of *Crambus ericella* and *Udea uliginosalis*, so late-flowering populations are unlikely to reproduce. The effect of climate change on the flowering time of orchids and activity of their pollinators is poorly known; however, previous studies indicate that global warming can lead to desynchronization and decline in the fruiting process of plants (Robbirt et al., 2014; Hutchings et al., 2018). Similar findings are reported by Tsiftsis and Djordjević (2020) for two deceptive species of the genus *Ophrys*, and they highlight a disruption of plant–pollinator interactions due to climate change, resulting in serious conservation consequences for these species. On the other hand, Molnár et al. (2012) reported that the phenology of nectar-rewarding orchids or short-lived species with non-Mediterranean distributions is less affected by global warming than that of autogamous or deceptive, long-lived species with mainly Mediterranean distributions. *Pseudorchis albida* belongs to the first group of species.

As the predicted changes in the ranges of the taxa studied differ, their future need of conservation is also likely to differ. *Pseudorchis albida* ssp. *straminea* is not threatened in the near future by changes in climate, whereas populations of *P. albida* ssp. *albida* are, especially in Central and Eastern Europe. Nevertheless, Pfeifer et al. (2010) indicated that relict areas are likely to occur in which this taxon can survive much longer than in new areas, which could be affected by various non-climate related factors. It is, therefore, best to maintain current populations in the best possible condition. Reinhammar et al. (2002) studied the population dynamics of *P. albida* over 6 years in two permanent plots (3 × 3 m), one mown and the other left to succession revealed that in the mown plot, the number of new individuals appearing annually was large and stable, whereas in the unmanaged plot, there was little or no recruitment. It is, therefore, important to maintain the stability of semi-natural habitats inhabited by *P. albida*.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

Author contributions

MK designed the research and collected data. AR performed statistical analyses. MK, AR, and SN defined the methodology and conducted the research, prepared figures, and wrote and reviewed the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2022.912428/full#supplementary-material

SUPPLEMENTARY DATA SHEET S1
Localities of *P. albida* used in analyses.

SUPPLEMENTARY DATA SHEET S2
Localities of *P. albida* pollinators used in analyses.

SUPPLEMENTARY DATA SHEET S3
Correlations between bioclimatic variables calculated using Pearson's correlation coefficient.

SUPPLEMENTARY DATA SHEET S4
Overlap of suitable niches for the orchids studied and their pollinators currently and in various climate change scenarios.

SUPPLEMENTARY TABLE S1
List of GBIF Occurrence Download used in the study.

SUPPLEMENTARY TABLE S2
Factor coordinates of the cases based on correlation.

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Taxonomic revision of *Sobralia* section *Racemosae* Brieger (*Sobralieae*, Orchidaceae)

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Sobralia Ruiz & Pav. is a large and morphologically diverse neotropical orchid genus. It can be divided into four sections and some informal groups of species based mainly on the inflorescence architecture. While most of the species have strongly abbreviated, compact raceme, the section *Racemosae* is characterized by an elongated inflorescence with distinct internodes between flowers. Although the group is well-defined and easily distinguishable in terms of morphology, its species are often similar to each other and may be easily misidentified. Identification is especially difficult when considering herbarium specimens. Here, a taxonomic revision of *Sobralia* section *Racemosae* is presented. Apart from particular species' morphological characteristics, keys for identification, ecological data, and distribution maps are presented. *Sobralia gambitana* is described as a species new to science. A neotype for *S. hoppii* Schltr. is proposed.

KEYWORDS

diversity, morphology, neotropics, new species, taxonomy

1. Introduction

Sobralia is a large orchid genus consisting of about 200 species distributed from southern Mexico to Brazil and Bolivia. Its representatives can be found in various habitats, from humid and shaded tropical forests to sunny, dry, open savannas or roadsides. They grow from sea level to over 3,000 m a.s.l. They can occur as terrestrial or lithophytic plants, but sometimes also as epiphytes (Pridgeon et al., 2006; Baranow, 2015).

Sobralia is a morphologically diverse group of species, especially when considering the architecture of inflorescence and morphology of floral bracts and flower segments. The differences allow distinguishing some groups of species, which served as the basis for the description of infrageneric units (Lindley, 1854; Reichenbach, 1873; Brieger, 1983).

The nominal section was characterized by lateral or rarely terminal inflorescences with branching, well-developed raceme and relatively small floral bracts compared to the size of the ovary (Brieger, 1983). The section *Racemosae* Brieger, despite terminal inflorescences, could be distinguished from the former by its elongated and unbranched inflorescences with large floral bracts. Section *Globosae* Brieger is composed of small plants with narrow leaf blades, small flowers positioned in the terminal, and condensed inflorescences (shortened internodes hidden under the floral bracts) that successively produce a single flower at a time and elongate with successively produced floral bracts. Species of section *Abbreviatae* Brieger share terminal and condensed inflorescences with the previous section but, instead, present floral bracts forming a cone. The fifth section, *Intermediae* Brieger, was established for a single taxon *Sobralia fragrans* Lindl. to emphasize its elongated basal internode of the inflorescences. Dressler (2002) enlarged this section, placing other species with small flowers and inflorescences.

The present classification of *Sobralia* is based on Briegers' 1983 division of the genus into sections. However, the development of molecular methods revealed that the nominal section of *Sobralia* is more closely related to other genera of *Sobralieae* than to the remaining groups of *Sobralia*. As the nominal section is also different in the morphological characters, such as branching and often lateral inflorescence, it was elevated to the rank of a separate genus *Brasolia* (Baranow et al., 2017, see also Dressler et al., 2011; Neubig et al., 2011).

Since then, the newly defined *Sobralia* consists of the species with terminal and unbranching inflorescences only. Most of the species have abbreviated and compact raceme, hidden between the floral bracts, forming a tight, cone-like structure, producing one or two flowers at a time. However, there is one group, section *Racemosae* Brieger, with elongated raceme, having distinct internodes. The flowers of its representatives develop from the nodes and are supported by distichous, large floral bracts. The inflorescence contains several flowers at various stages of growth, with the youngest ones on its top. The distinct morphology is supported by the results of the molecular study, which can be seen in the phylogenetic trees (Neubig et al., 2011; Baranow et al., 2017). Also, the karyotype evolution analysis with the phylogenetic study as the background (Baranow et al., 2022) as well as niche conservatism and ecological tolerance evolution study (Kolanowska et al., 2022) have confirmed the consistency of the group. Thus, the section appears to be well-defined and distinct from the other groups of the genus. On the other hand, the species of *Racemosae* are in many cases similar to each other and easy to misidentify. The only study devoted to *Racemosae* was made by Romero-González (2003), but the author focused only on *S. liliastrum* Lindl. and its close allies.

The study aims to present the results of the taxonomic revision of all species of the section *Racemosae* with the descriptions and illustrations of their morphology, with the ecological data and maps of distribution. The revision of the herbarium material resulted in a discovery of the collection, which, in order of its distinctness, was recognized as a species new to science. Additionally, a neotype for *S. hoppii* Schltr. is selected. In addition, the first comprehensive key for the identification of the species of section *Racemosae* is provided.

2. Materials and methods

The presented revision was based on the morphological study of the herbarium material deposited in the following herbaria AMES, BM, COAH, COL, CUVC!, F, K, K-L, MO, NY, P, UGDA-DLSz, U, US, W, W-R (Thiers, 2022). In total, over 440 herbarium specimens were examined within the study.

Apart from the morphological data, the herbarium specimens were also a source of information concerning the ecology of the studied species given under the morphological descriptions. Moreover, the localities of the collections were used for the distribution presentation, and the geographical distribution maps were generated using the software QGIS version 3.22.12[1] and the Natural Earth[2] data.

A conservation analysis was performed using the criteria from the International Union for the Conservation of Nature (IUCN, 2022). The Extent of Occurrence (EOO) and the Area of Occupancy (AOO) of each species were estimated using GeoCat (Bachman et al., 2011).

3. Results

3.1. *Sobralia* Ruiz & Pav. section *Racemosae* Brieger

Orchideen **1** (13): 798. 1983; Type species: *Sobralia rosea* Poepp. & Endl., Nov. Gen. Sp. Pl. **1**: 54, t. 93. 1836.

The group contains 15 species occurring in South America with the greatest species diversity in Northern Andes.

3.1.1. Key to the species

1. Leaves less than 5 cm wide . . . **2**

2. Flowers deep rose–purple with bright yellow throat of the lip, apical stelidia of gynostemium not exceeding anther apex . . . **1. *S. paradisiaca***

2* Flowers yellow or white with yellow lip disk, apical stelidia of gynostemium long, strongly exceeding anther apex . . . **3**

3. Flowers yellow, stelidia rounded at apex . . . **2. *S. chrysantha***

3* Flowers white or navy yellow with yellow or orange lip disk or a dot on the apical part, stelidia acute at apex . . . **4**

1 www.qgis.org/pl/

2 www.naturalearthdata.com

4. Lip divided into basal and apical parts by the distinct constriction just below the middle, gynostemium stelidia, horn-like, falcate . . . **3. *S. chrysoleuca***
4* Lip not constricted in the middle, gynostemium stelidia narrowly oblong **5**
5. Leaves ca 4 cm wide . . . **6. *S. liliastrum***
5* Leaves up to 2.5 cm wide . . . **6**
6. Lip white with yellow throat and reddish orange elevated keels, floral segments 50–65 mm long . . . **4. *S. elisabethae***
6* Lip hyaline white with pale yellow, elevated keels, floral segments 40–45 mm long . . . **5. *S. granitica***
1* Leaves 6–12 cm wide . . . **7**
7. Rachis fractiflex, bracts horizontally spreading, acute or obtuse . . . **8**
8. Flowers white, lip red–purple on the lamina and the throat, floral segments 70–75 mm long, lip furnished with a pair of shallow ridges in the throat only . . . **7. *S. luerorum***
8* Flowers creamy white with purple striation on lip, floral segments up to 60 mm long, lip with two basal ridges and 5–7 parallel lamellae running from the base to the apex . . . **8. *S. gloriosa***
7* Rachis sinuously flexuous, bracts suberectly spreading, acuminate . . . **9**
9. Floral bracts leaf-like, up to 20 cm long, decreasing in size toward the apex of inflorescence . . . **9. *S. ruckeri***
9* Floral bracts up to 12 cm . . . **10**
10. Floral segments not exceeding 90 mm in length, lip with two basal ridges, additional lamella can be present too, but it is restricted to the middle of the lip only . . . **11**
11. Two basal thickenings fused together except their margins . . . **10**. ***S. gambitana***
11* Two basal thickenings separate . . . **12**
12. Lip without any protuberances apart from the basal lamellae . . . **11. *S. tamboana***
12*. Lip with thickenings or lamellae running along one or more central veins . . . **13**
13. Lip base with 2 lamellae running to its middle and the central vein in central part ornamented with lamella . . . **12. *S. splendida***
13* Lip with 2 basal keels, median vein thickened, with two additional thickenings near the middle . . . **13. *S. hoppii***
10* Floral segments 100 mm or more, lip disk with 3–7 lamellae running from the base up to at least its middle . . . **14**
14. Lip white with a broad white margin, with purple veins in the center, disk with 3 lamellae running from the base to the middle, the median-one high-carinate . . . **14. *S. pulcherrima***
14* Lip dark purple–magenta with very narrow, white margin, in center with fine, radiating, white veins, disk from the base to center transversed by 5–7 low, parallel lamellae . . . **15. *S. rosea***

3.1.1.1. *Sobralia paradisiaca* Rchb.f.

Linnaea **22**: 816. 1850. Type (designated by Baranow *in* Szlachetko et al., 2020: 248): Venezuela. Merida. *Sine prec loc*. Alt. 1600 m. March 1847. N. *Funk and L.J. Schlim 1489* (Lectotype: W!, Isolectotypes: K!, P!).—Garay and Dunsterville. Venezuelan Orchids Illustrated 404. 1959.—Szlachetko et al. Materials to the Orchid Flora of Colombia **3**: 248. 2020.

Plants up to 130 cm tall, caespitose, often leafy for all except the basal quarter. Stem concealed in green tubular sheathing leaf bases which tend to become red or dark red when well exposed. Leaves up to 25 cm long and 4.5 cm wide, lanceolate, apex lightly attenuate, plicate, the sides of blades tend to be revolute, making the upper surface convex, the uppermost leaves smaller than the ones below, with spathe-like base subtending the rachis. Inflorescence producing 3–6 flowers developing in succession from 1 to 3 at a time; rachis terete, fractiflex. Sepals and petals deep rose–purple paler right at base, lip deep rose–purple with bright yellow throat. Dorsal sepal up to 65 mm long and 25 mm wide, oblanceolate to ligulate-oblanceolate, acute, moderately fleshy. Lateral sepals up to 70 mm long and 33 mm wide, ligulate-lanceolate, somewhat oblique, moderately fleshy. Petals up to 70 mm long and 30 mm wide, elliptic-oblanceolate, acute, somewhat oblique. Lip 48–70 mm long, 33–50 mm wide when spread, elliptic-rectangular in general outline, entire, apical margins truncate, strongly undulate and crispate, thin for the most part but axially much thickened at base where there are two ventral swellings about 10 mm long, projecting from each side and almost touching each other, the rest of the axial part not thickened but with several raised veins giving the impression of a thickening terminating in a small hollow point. Gynostemium up to ca 35 mm long, stelidia short, obscure, subequal in length to the anther or shorter (**Figure 1**).

Ecology: Terrestrial. Flowering in March, September, and December.

Distribution: Colombia, Venezuela. Alt. 1600–2300 m.

Conservation status: EOO—CR, AOO—CR.

Representative specimens (**Supplementary Map 1**)—**Venezuela**. Merida. Between La Carbonera and La Azulita. 17 September 1966. *J. de Bruijn 1134* (MO!); *Sine loc.* Alt. 2300 m. *H. Wagener 124* (W! 21607, UGDA-DLSz!–drawing). **Colombia**. Norte de Santander. Ocaña. Alt. 1830 m. 1846. *L.J. Schlim 1203* (W-R!). Vaupés. Entre Wacaricuara y El Varador. Al Río Yi. 9–12 December 1952, *R. Romero Castañeda 3922* (COL!).

Sobralia paradisiaca belongs to the group of species having relatively narrow leaves (up to 5 cm width) along with *S. chrysantha*, *S. liliastrum*, *S. chrysoleuca*, *S. elisabethae*, and *S. granitica*. It can be easily separated from all of them by the color of the flowers—it is the only taxon having deep rose–purple tepals with a bright yellow throat of the lip. The species differs from other *S. liliastrum*-complex representatives also by very short, rounded stelidia of gynostemium.

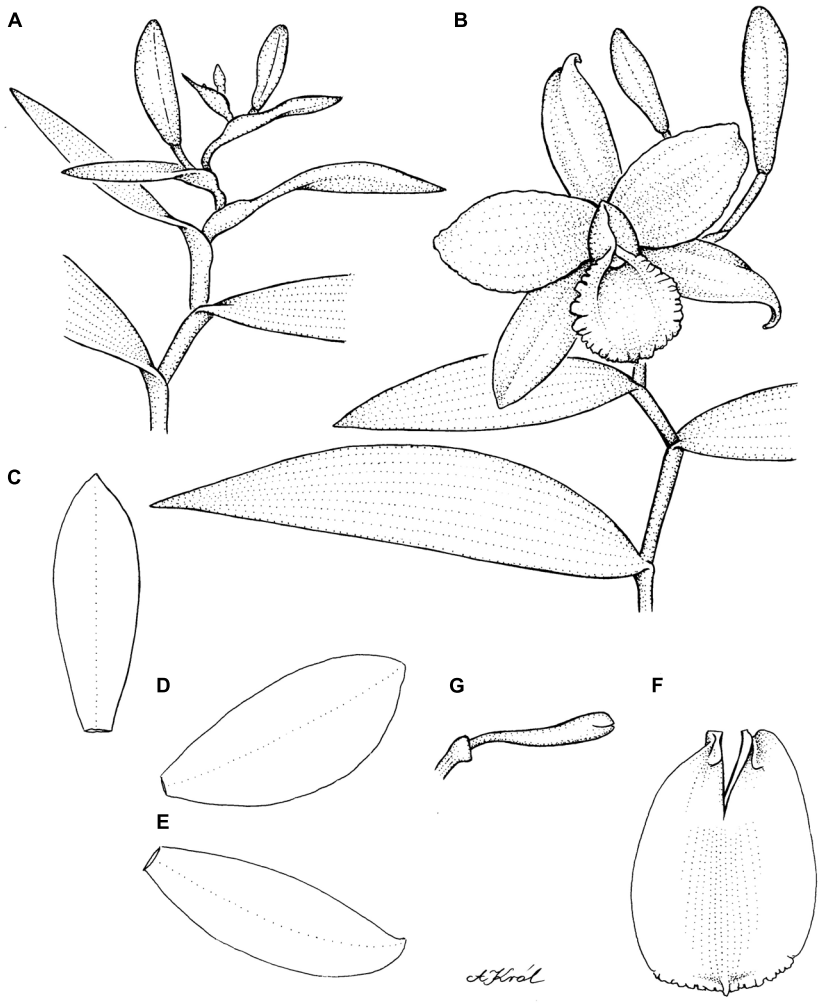

FIGURE 1
Sobralia paradisiaca Rchb. f. **(A,B)** apical part of stem with the inflorescence, **(C)** dorsal sepal, **(D)** petal, **(E)** lateral sepal, **(F)** lip, and **(G)** gynostemium [redrawn by A. Król from Dunsterville and Garay (1959)].

In our opinion, there is a mistake in the protologue of the species. In W and K there are *Funck and Schlim* collections numbered 1489, and not 1749 as stated in the original description. Apart from fragments of plants, the collection includes also a hand drawing of a plant and floral parts (W-R 21609). It appears that collection 1489 should be indicated as the type specimen.

Dunsterville and Garay (1959) stated that *S. paradisiaca* may be only a juvenile form of *S. liliastrum* and treated as a synonym of the latter species. Surprisingly, in the same publication, the same authors listed *S. paradisiaca* as a valid species emphasizing its distinctness observed during the study of the type specimen.

3.1.1.2. *Sobralia chrysantha* Lindl.

Fol. Orchid. **5** (Sobralia): 3. 1854. Type: Colombia. (Santander). Socorro. Alt. 1220 m. *L.J. Schlim 6* (Holotype: K-L!, ISOTYPE: W!).—Szlachetko et al. Materials to the Orchid Flora of Colombia **3**: 249. 2020.

Plants height unknown, probably well over 100 cm tall. Leaves up to 25 cm long and 4 cm wide, lanceolate to elliptic-lanceolate, acute, plicate. The leaf subtending the rachis up to 10 cm long. Inflorescence ca 10 cm long, rachis inconspicuously flexuose. Floral bracts 15–50 mm long, narrowly lanceolate-triangular, acute to acuminate. Flowers yellow, large. Dorsal sepal 83 mm long, 12 mm wide, oblong-ligulate to linear, subobtuse. Lateral sepals 83 mm long, 12 mm wide, obliquely linear-lanceolate, shortly acuminate. Petals 85 mm long, 13 mm wide, obliquely linear-lanceolate to ligulate-lanceolate, shortly acuminate. Lip 70 mm long, up to 49 mm wide, broadly obovate to suborbicular-obovate in outline above cuneate base, rounded at apex, indistinctly denticulate and undulate along margins in the upper half, attenuate and canaliculate

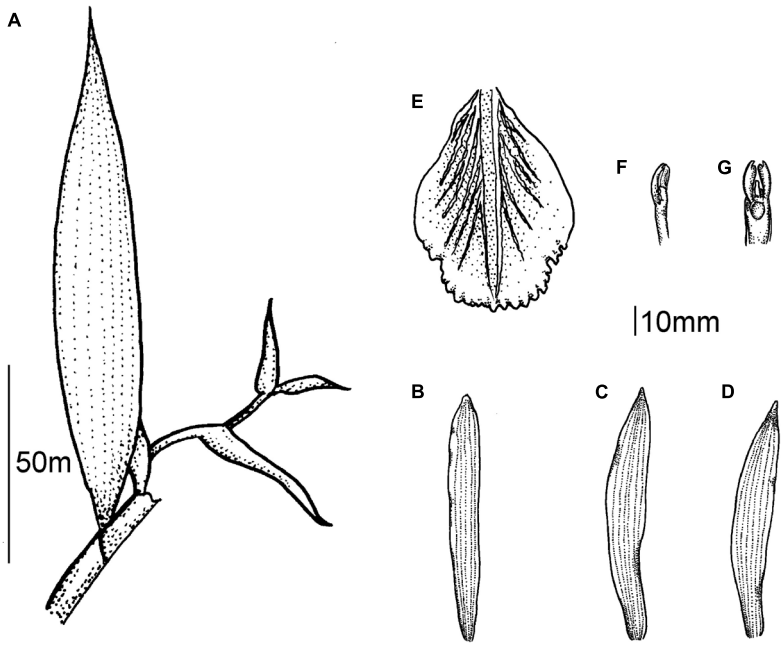

FIGURE 2
Sobralia chrysantha Lindl. **(A)** apical part of stem with inflorescence, **(B)** dorsal sepal, **(C)** petal, **(D)** lateral sepal, **(E)** lip, **(F)** apical part of gynostemium, side view, and **(G)** apical part of gynostemium, front view [drawn by P. Baranow; panels **(A,E–G)** from the isotype, lip, and gynostemium redrawn from the drawings left on the herbarium sheet; panels **(B–D)** from *Schlim 1188*].

toward base, without thickenings except the still middle rib. Gynostemium 52 mm long, clavate, staminodes oblong elliptic, straight, much exceeding the anther, with a deep wing at their back and an oblique emargination, apex falcate, blunt (Figure 2).

Ecology: Terrestrial. No data on flowering time.

Distribution: Colombia. Alt. 1220–2000 m.

Conservation status: EOO—CR, AOO—CR.

Representative specimens (Supplementary Map 2)–**Colombia**. Santander. Socorro. Alt. 1300–2000 m. 1849. *L.J. Schlim 1188* (W!, UGDA-DLSz!–drawing); Socorro. Alt. 1220 m. *L.J. Schlim 6* (K-L!).

It is interesting to note that Reichenbach's drawing accompanying the type specimen stored at W shows a very massive stelidia which are apically bilobed, with the anterior lobe being somewhat longer and acute, and the posterior one shorter and rounded. In the materials examined we did not find stelidia of this form.

Sobralia chrysantha resembles *S. liliastrum*-complex in habit and with a very long stelidia much exceeding the anther apex but can be easily distinguished by the color of the flowers (yellow vs. white in *S. liliastrum* and its allies) and rounded apex of stelidia (vs. acute in *S. liliastrum* complex).

3.1.1.3. *Sobralia chrysoleuca* Rchb. f.

Xenia Orchid. 2: 179. 1873. Type: BOLIVIA. *Sine loc. S.A. Pearce 777* (Holotype: W! 21594, UGDA-DLSz!–drawing).

Erect plant, height unknown, probably well above 100 cm tall. Leaves 30 cm long and 4 cm wide, oblong lanceolate to linear-lanceolate, acuminate, coriaceous, strongly plicate. Inflorescence 12 cm long, ca. 15-flowered, rachis erect, nearly straight to somewhat flexuose. Floral bracts 25–30 mm long, triangular-lanceolate, acuminate. Ovary 30 mm long. Flowers white or light yellow with distinct, deep yellow or orange dot on the apical part of lip. Dorsal sepal 68 mm long, 18 mm wide, lanceolate, acute. Lateral sepals 60 mm long, 15 mm wide, oblong-lanceolate, inconspicuously oblique, acute. Petals 57 mm long, 25 mm wide, widely oblong or elliptic, somewhat oblique, acute. Lip 60 mm long, 45 mm wide, oblong, constricted below the middle and inconspicuously bilobed at the apex, margins in apical part irregularly crenate and crispate, disk with nine keels running along the central veins from base almost to the apex, base papillate. Gynostemium 36 mm long, slender but with large and wide, massive, wing-like, triangular, oblique, acute apical stelidia, which distinctly exceeding the anther apex (Figure 3).

Ecology: Terrestrial.

Distribution. Bolivia.

Representative specimens–**BOLIVIA**. *Sine loc. S.A. Pearce 777* (W!, UGDA-DLSz!–drawing).

Unique characters of this species are lip constricted near the middle, not found anywhere in the section *Racemosae*, and massive, horn-like, falcate stelidia.

According to the note on the herbarium label of the type specimen, the flowers of the species may be white and yellow

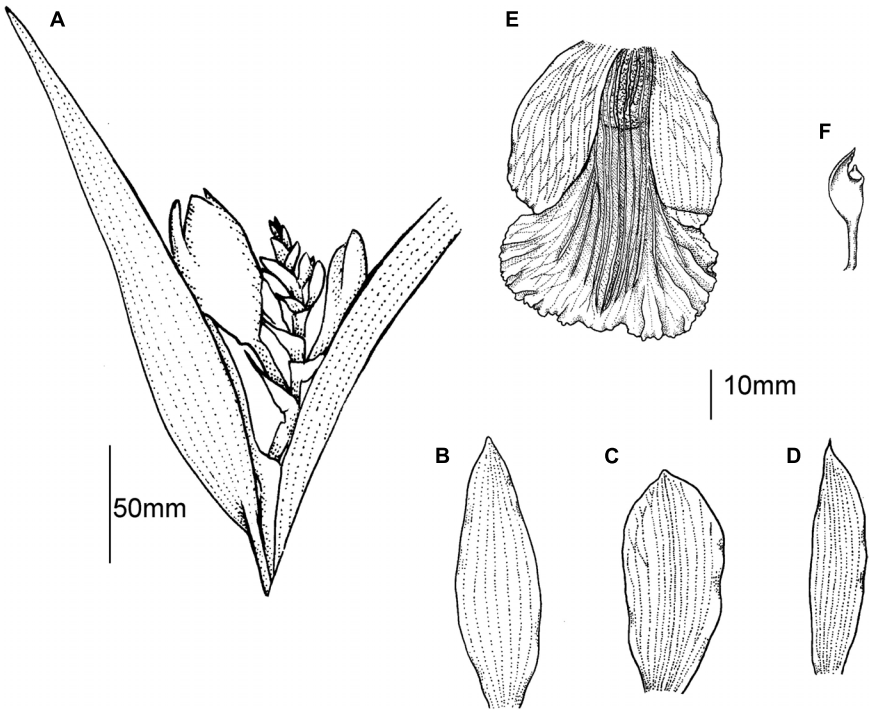

FIGURE 3
Sobralia chrysoleuca Rchb. f. **(A)** apical part of stem with inflorescence, **(B)** dorsal sepal, **(C)** petal, **(D)** lateral sepal, **(E)** lip, and **(F)** gynostemium (drawn by P. Baranow from the holotype).

colored. It may explain why the author compared the taxon with *Sobralia aurantiaca* (a synonym of *S. infundibuligera* with a compact inflorescence, hidden between the bracts, not similar to those of the section *Racemosae* representatives)—the taxa are similar in order to the flower color.

3.1.1.4. *Sobralia liliastrum* Lindl.

Gen. Sp. Orchid. Pl.: 177. 1833. Type (designated by Baranow and Szlachetko, 2016: 339): Brazil. Bahia. *P. Salzmann s.n.* (Lectotype: K! 000293880–plant on the right side of herbarium sheet; Isolectotypes: K!, MO!, W-R!, NY!—photograph, UGDA-DLSz!–drawing). ≡ *Cattleya liliastrum* (Lindl.) Beer, Prakt. Stud. Orchid.: 212. 1854.—Garay and Dunsterville. Venezuelan Orchids Illustrated 322. 1959.–Baranow and Szlachetko, Pl Syst Evol. **302**: 338. 2016.—Szlachetko et al. Materials to the Orchid Flora of Colombia **3**: 250. 2020.

= *Sobralia liliastrum* var. *alba* Lindl., Fol. Orchid. **5** (Sobralia): 4. 1854. Type: not designated

= *Sobralia liliastrum* var. *rosea* Lindl., Fol. Orchid. **5** (Sobralia): 4. 1854. Type: not designated.

= *Sobralia liliastrum* f. *maior* Hoehne, Relat. Commiss. Linhas Telegr. Estratég. Matto Grosso Amazonas 5, Bot. **4**: 23, pl. 74. 1912; Type: not designated.

Plants up to 300 cm tall, caespitose, erect, terete, the base with the remains of sheaths, the apex leafy, perfectly smooth, light green. Leaves up to 22 cm long and 4 cm wide, lanceolate to narrowly lanceolate, long acuminate, prominently veined on the underside, plicate. Inflorescence terminal, elongate, racemose, fractiflex, laxly few-flowered. Flowers produced in succession, large, white, lip with yellow throat. Dorsal sepal 58–70 mm long, 11.5–15 mm wide, oblong oblanceolate, acuminate, somewhat fleshy, and thick. Lateral sepals 58–70 mm long, 11.5–15 mm wide, oblong oblanceolate, acuminate, subfalcate, somewhat fleshy and thick, lightly carinate dorsally. Petals 55–70 mm long, 16–21 mm wide, oblong elliptic, subobtuse to subacute, slightly falcate, thin, finely sulcate dorsally. Lip 60–62 mm long, 43–54 mm wide, suborbicular-subflabellate in general outline, widest above the middle, obscurely 3-lobed, truncate at the apex, the apical margin erose, soft, thin, more or less undulate, sometimes with obscurely keeled lateral veins, often papillate at the base. Gynostemium 48 mm long with apical lanceolate-subfalcate, acute stelidia much exceeding the anther apex.

Ecology: Terrestrial along lowland rivers, in savannas, and on steep embankments with subxerophytic plants. Flowering throughout the year (**Figure 4**).

Distribution: Venezuela, Guyana, Suriname, French Guiana, Brazil. Alt. up to 2255 m.

Conservation status: EOO—LC, AOO—EN.

Representative specimens (**Supplementary Map 3**)—**Colombia**. Amazonas. Araracuara. Sabana de la Angostura. Alt. 400 m. 21 December 1951. *H. Garcia Barriga and R.E. Schultes*

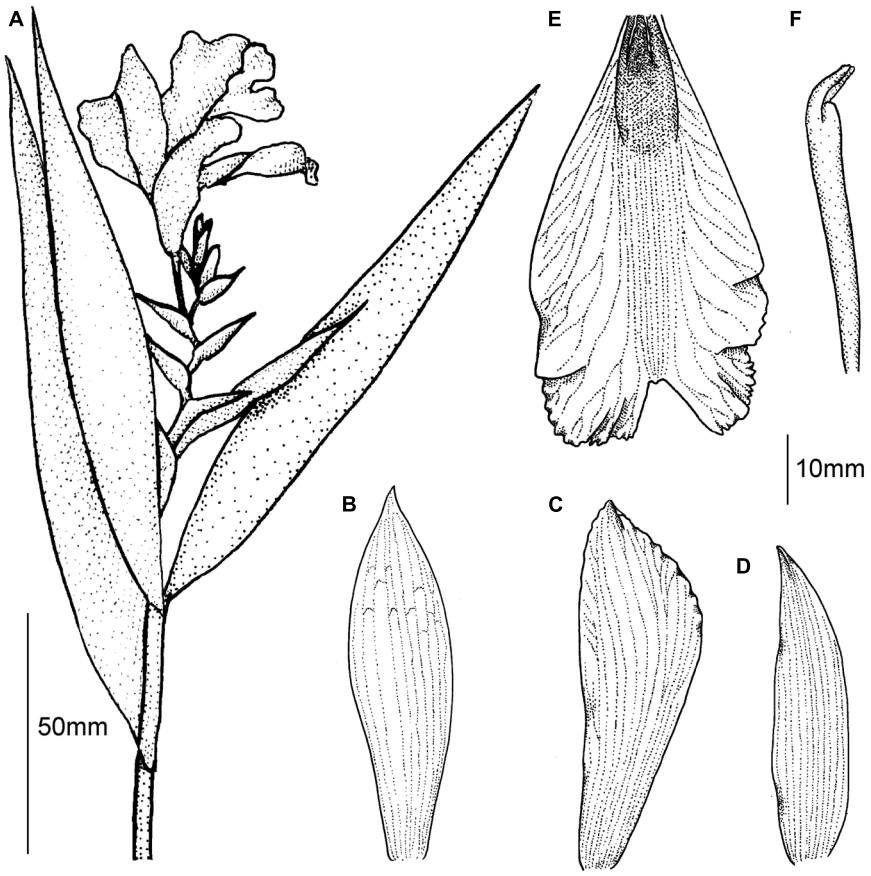

FIGURE 4
Sobralia liliastrum Lindl. **(A)** apical part of stem with inflorescence, **(B)** dorsal sepal, **(C)** petal, **(D)** lateral sepal, **(E)** lip, and **(F)** gynostemium, side view (drawn by P. Baranow from the isolectotype kept at MO).

14143 (COL!); Río Caquetá. La Pedrera. Cerro de Cupati. Alt. 240–580 m. 30 September 1952. *H. Garcia Barriga 14529* (COL!); Corregimiento La Pedrera, comunidad Bocas del Pira, sabana "Vasewai," margen derecha del Río Apaporis, approx. 10 min en bote de la comunidad Bocas del Pira Río arriba, resguardo Yaigoje-Apaporis. 0°27′05″S, 70°14′40″. Alt. 240 m. 31 March 2009. *J. Betancur, D. Cardenas, D. Tanimuca, and E. Tanimulka 13995* (COL!); Araracuara. Río Caquetá. 1 April 1976. *C. Sastre and H. Reichel D. 5182* (COL!). Caquetá. Sierra de Chiribiqueta, Campamento Sur. Al. SW del Campamento, entre este y los primeros de la meseta. 0°55′N, 72°45′W. Alt. 350–400 m. 7 July 1990. *P. Franco, J. Estrada, J. Fuertes and P. Palacios 3237* (COL!, US!); Sierra de Chiribiquete. Mesa encima de la Cueva de las Pinturas, 1°05′N, 72°40′W. Alt. 740–760 m. 21 August 1992. *P. Palacios 2417 and P. Franco, O. Rangel, and J. Betancur* (COL!–sterile); Sierra de Chiribiquete. Campamento Norte. Prox. del campamento. 1°7′N, 72°50′W. Matorrales de sabana. 6 December 1990. *J.M. Cardiel, S. Castroviejo, G. Galeano and F. Gonzalez 1010* (COL!); Sierra de Chiribiquete. En la *via* del Campamento a la Cueva de Pinturas. 1°05′N, 72°40′W. Alt. 600 m. 17 August 1992. *P. Franco 3718 and O. Rangel, P. Palacios, and J. Betancur* (COL!). Chocó. Alrededores de Coredo. 22 October 1946. *R. Romero Castañeda 519* (COL!). Guainia. Poblacion el Remanes. Cerros de Mavicure y Pajarito a orillas del Río Inírida, 40 km de Puerto Inírida. 1978. *F. Sarmiento 1084* (COL!); Correg. de San Felipe, Río Negro. Alrededores de la pista de aterrizaje. Alt. 100 m. 28 September 1977. *M. Pabon E., J. Espina, and C. Dominguez 228* (COL!); Caserio de Sta Rita, Río Guainia. Alt. 100 m. 15 October 1977. *M. Pabon E., J. Espina, and C. Dominguez 337* (COL!). Guaviare. Mesa La Lindosa, Cerrito a 15–20 km al S de San José del Guaviare. Alt. 400–600 m. 13–15 December 1950. *J.M. Idrobo and R.E. Schultes 656* (COL!); Mpio. San José del Guaviare. Carretera de San José a Puerto Arturo, km 3, alrededores de la finca Santa Gertrudis, 2°28′20″N, 72°41′30″W, Alt. 280 m. 21 January 1996. *R. Lopez and O.J. Rodriguez 976* (COAH!, MO!); San José del Guaviare. Antiqua represa. Alt. 200–250 m. 27 December 1993. *C. Sastre and J.P. Robin 9194* (COL!); San José del Guaviare. Ciudad Perdida o Ciudad de Piedra. Alt. 250–300 m. 28 December 1993. *C. Sastre and J.P. Robin 9218* (COL!). Meta. Serrania de La Macarena, margen izquierda del Río Guayabero, a 10 km abajo de Caño Lozada. Alt. 500 m. 16

January 1959. *P. Pinto E., H. Bischler, and R. Jaramillo M. 206* (COL!, P!); Reserva Nacional de la Macarena, southernmost slope of Macarena Mts, immediate to the Río Guayabero. Alt. 250–300 m. 25 January 1968. *J. Thomas, J. Hernandez C., and P. Pinto E. 1589* (P!). Vaupés. Río Macu-Parana, tributary of the Río Papuri. 8 August 1943. *P.H. Allen 3047* (COL!); Yapoboda, 10 December 1943. *P.H. Allen 3224* (MO!); Bacuraba Cachoeira (the first major cataract on the Vaupés East of Mitú). Alt. 200 m. 4 November 1944. *P.H. Allen 3311* (MO!); Env. of Río Mitú, dry arud slopes of the Cerro of Mitú, El Cerro de Guacamaya, 30 October 1976. *E.W. Davis 201* (COL!, U!); Río Pira Paraná (tributary of Río Apaporis, between 0°15′S, 70°30′W and 0°25′N, 70°30′W, 6 September 1952. *R.E. Schulters and I. Cabrera 17232* (U!, US!, UGDA-DLSz!–copy); Yurupari, orilla Vaupés, 350 km arriba de Mitú. Alt. 220 m. 24 September 1939. *J. Cuatrecasas 6961* (COL!); Río Vaupés, cachivera de Yurupari. Alt. 400 m. 24–26 October 1952. *H. Garcia Barriga 14935* (COL!); Río Vaupés, Mitú and vicinity. September–October 1966. *R. Schultes 24344* (COL!); Mitú and vicinity. Río Parana-Pichuna, savanna at major rapids, 6 September 1976. *J.L. Zarucchi 1957a* (COL!); Desembocadura del Ariari con el Río Guayabero. Cabana del Incora "Bocas del Ariari," 21 February 1969. *P. Pinto E. and C. Sastre 942* (COL!, P!); Vicnity of Mitú. Trail to Cerro Mitú. Caatinga forest. Alt. 200–250 m. 2 October 1991. *J. Kress, J. Betancur, C. Roesel, and R. Echeverry 91-3336* (COL!); Río Vaupés, Cerro de Circasia, entre el Río Ti y Namu. Alt. 380–450 m. 30 October 1952. *H. Garcia Barriga 15028* (COL!); Río Kubiyu, Cerro de Canenda. Alt. 380–680 m. 2–4 November 1952. *H. Garcia Barriga 15074* (COL!); Caño Cubiyú. Comunidad Indigena La Sabana. 1°15′N, 70°51′W. Alt. 200 m. 26 April 1993. *S. Mandrinan, G. Ngan, and J. Page 1175* (COL!, NY!); Riberas del Río Inírida (69°45′W), sitio Raudal Alto o Mariapiri, margen derecha. Alt. 180 m. 3 February 1953. *A. Fernandez 2121* (COL!); Cerro Mitú. Alt. 400–450 m. 4 September 1959. *B. Maguire, C.K. Maguire, and A. Fernandez 44097* (COL!); Río Kuduyari. Yapoboda, sandstone savanna near headwaters. 5 October 1951. *R. E. Schultes and I. Cabrera 14243* (COL!); The same loc. 18 November 1952. *R.E. Schultes and I. Cabrera 18497* (COL!); Serrania de Taraira. 10 km al NW del raudal de la Libertad. 0°53′, 69°45′W. Bosque de caatinga. Alrededores del campamento. Alt. 250 m. 31 August 1993. *J. Rodriguez 183* (COL!); Serrania de Taraira. 10 km al NE del raudal de la Libertad. 0°58′S, 69°45′W. Alt. 250 m. 2 August 1993. *R. Cortes and J. Rodriguez 764* (COL!); Cerro de Chiribiquete, a un lado del Río Macaya, terreno muy pedregoso. 17 January 1944. *G. Guiterrez and R.E. Schultes 683* (NY!). Vichada. Parque Nacional Natural, "El Tupparo," on granitic outcrops between the mouth of the Río Tupparo to Raudal Maipures alon the Río Orinoco, 5°12′N, 67°50′W. Alt. 90–130 m. 1 March 1985. *J.L. Zarucchi and C.E. Barbosa 3521* (MO!). **Venezuela**. Amazonas. Río Sipapo entre Isla Lencho y Boca del Cuao. Mpio Autana, 4°54′–5°3′N, 67°34′–67°46′W. 28 January 1997. *A. Castillo 4474* (MO!); Dpto Atabapo, Sabana Graminosa arbustiva en altiplanicie (Cerro Paru), 4°34′N, 65°31′W. Alt. 590 m. February 1992. *A. Chaviel 205* (MO!); Dpto Atures, Serrania de la Coromoto, Sector "El Tobagin," a 37 km al. S de Pto. Ayacucho. 5°24′N, 67°35′N. Alt. 80–200 m. 19 January 1989. *N. Cuello 344* (MO!); Dpto Atabapo, Zona de Lomerio con Sabana Arbustiva y Altiplanicie con Herbazal Subarbustivo Tepuyano. 3°33′N, 64°29′W. Alt. 1400 m. November 1991. *Y. Fernandez and M. Yanez 856* (MO!); Por debajo del Salto Remo, 110–71 km por arriba del Guayapo. 4°34′N, 67°18′W. Alt. 120 m. May 1989. *E. Foldats and J. Velazco 9462* (MO!); Dpto Atabapo, Alto Río Orinoco, 15 km al. W de la Esmeralda, Cerro Baraco. 3°8′N, 65°41′W. Alt. 300 m. 1 March 1990. *G.G. Aymard and L. Delgado 8283* (MO!); 9 km northeast of San Carlos de Río Negro. 1°57′N, 67°3′W. Alt. 120 m. 25 November 1977. *R.L. Liesner 3582* (MO!); 10 km NE of San Carlos de Río Negro. 1°54′N, 67°00′W. Alt. 120 m. 28 January 1980. *R.L. Liesner 8830* (MO!); 2 km east of San Carlos de Río Negro. 1°55′N, 67°5′W. Alt. 120 m. 13 November 1977. *R.L. Liesner 3421* (MO!); 10 km NE of San Carlos de Río Negro, (ca. 20 km S of confluence of Río Negro and Brazo Casiquiare), 1°56′N, 67°03′W. Alt. 120 m. 24 April 1979. *R.L. Liesner 6947* (MO!); Atures, Río Coro-Coro, W of Serrania de Yataje, 6–8 km N of settlement of Yutaje, 5°41′00″N, 66°07′30″W. Alt. 320 m. 23 February 1987. *R.L. Liesner and B. Holst 21326* (MO!); Dpto Atures, 1 to 2 km E of Río Coro-Coro, W of Serrania de Yataje, 8 km N of settlement of Yutaje, 5°41′30″N, 66°07′30″W. Alt. 600–650 m. 25 February 1987. *R.L. Liesner and B. Holst 21383* (MO!); "El Tobogan de la Selva," 35 km south of Puerto Ayacucho. Alt. 85 m. 21 February 1979. *T. Plowman 7702* (F!); Caño Cupaven, Río Orinoco at mouth of Río Atabapo. Alt. 150 m. 11 May 1954. *J. Silverio Level 82* (F!, MO!); Camino San Carlos de Río Negro-Solano, 10–22 February 1989. *B. Stergios, K. Kubitzki, G. Aymard, and E. Melguiero 13396* (MO!, US!); Río Negro, Piedra Ignea, Cerro Aratityope, 2°10′N, 65°34′W, approx. 70 km al SSW de Ocamo, con richuelos afluente al Río Manipitare. Alt. 990–1670 m. 24–28 February 1984. *J.A. Steyermark, P. Berry, and F. Delascio 130051* (U!); Río Negro, piedra ignea, Cerro Aratitiope, approx. 70 km al SWW de Ocamo, 2°10′N, 65°34′W. Alt. 990–1670 m. 24–28 February 1984. *J.A. Steyermark, P. Berry, and F. Delascio 130051* (MO!); Dept. Atabapo, Cerro Duida. 3°40′N, 65°45′W. Alt. 1400 m. 10 February 1982. *J.A. Steyermark, M. Guariglia, N. Holmgren, J.L. Luteyn, and S. Mori 126433* (MO!, K!); Atabapo, sabanas y bisques ubicados al pie nor-oriental y oriental del Cerro Cucurito, ribera SE del medio Caño Yagua. 3°36′N, 66°34′W. Alt. 120 m. 8 December 1978. *O. Huber and S.S. Tillett 2941* (K!, U!); Bolivar. Roscio, 3 km S of El Pauji. 4°30′N, 61°35′W. Summit of mountain bordering N side of "El Abismo," thick low rocky scrub. Alt. 1050 m. 19 October 1985. *B.K. Holst and R.L. Liesner 2355* (MO!, U!); Río Negro, Slope of Cerro Aracamuni. Aracamuni. Quebrade Camp, in area of rapids flowing over laja (stone), 1°24′N 65°38′W. Alt. 600 m. 20 October 1987. *R.L. Liesner and F. Delascio 22240* (MO!, U!);

Transecta entre conucos al. E de Santa Rosa de Ucata, passando por bosque humedo, hast arbustal de arena blanca al. E de este pobiado, 4°24′N, 67°46′W. Alt. 80–85 m. 23 October 1989. *G.A. Romero and E. Melguiero 2235* (K!, MO!); Cerro granitico al. E del Raudal Gavilan, caminando ca 2 horas desde la parcel. 5°37′N, 67°22′W. Alt. 100 m. 1 February 1991. *G.A. Romero, E. Melgueiro, and C. Gomez 2291* (MO!); Laja granitica al. E del Raudal Gavilancito, vegetation en pequenas depresiones y grietas en la piedra, 5°37′N, 67°22′W. Alt. 80–100 m. 9 February 1992. *G.A. Romero, E. Melgueiro, and C. Gomez 2365* (MO!); Esmeralda Ridge, between Esmeralda and base of Cerro Duida. Alt. 150 m. 21 August 1944. *J.A. Steyermark 57744* (F!); Atabapo, Boca de Mesaque. 3°04′N, 67°06′W. Alt. 80 m. November 1989. *J. Velazco 953* (MO!). Bolivar. Along highway between Santa Elena and Icabaru 103 km SW of Santa Elena, 16 km NE of Icabaru, near bridge. 4°20′N, 62°45′W. Alt. 750 m. 24 July 1982. *T.B. Croat 54045* (MO!); By main road, ca 11 kms E of Kavanayén. Alt. 1200 m. 26 July 1983. *R. Kral Wit and A.C. Gonzalez 70462* (MO!); Gran Sabana, ca 15 km WSW of Karaurin Tepui, Quebrada Tanuan. 5°19′N, 61°04′W. Alt. 950 m. 1 May 1988. *R.L. Liesner 24119* (MO!); 17 km E of El Pauji by road and 64 km W of Santa Elena by road, 4 km N of highway. Río Las Ahallas, 4°30′N, 61°30′W. Alt. 850 m. 29 October 1985. *R.L. Liesner 19122* (MO!); 3 km S of El Puaji, Morichal, 4°30′N, 61°35′W. Alt. 900 m. 19 October 1985. *R.L. Liesner and B.K. Holst 18811* (MO!); 17 km E of El Pauji by road and 64 km W of Santa Elena by road, 4 km N of highway. Río Las Ahallas, 4°30′N, 61°30′W. Alt. 850 m. 1 November 1985. *R.L. Liesner 19311* (MO!); Sabana de Arekuna, E margin of lower Río Caroni. 6°31′N, 62°53′W. Alt. 520 m. 29 August 1983. *G.T. Prance and O. Huber 28316* (MO!); N de Raudalito, Río Sipapo. Alt. 120 m. 10 October 1988. *G.A. Romero and F. Guanchez 1631* (MO!); Km 146 al. sur de El Dorado. Alt. 1280 m. 15–18 November 1978. *J.A. Steyermark, J.L. Luteyn and M.L. Lebron-Luteyn 117553* (MO!); Gran Sabana, between Mission of Santa Teresita de Kavanayén northwest to Río Karuai, on large mes. Alt. 1220 m. 26 October 1944. *J.A. Steyermark 59387* (F!); Sororopan tepui, crest of cerro between east and west end. Alt. 2255 m. 14 November 1944. *J.A. Steyermark 60117* (F!). **Guyana**. Upper Mazurani River Region. Karowtipu Mountain. 5°45′N 60°35′W. Alt. 1000 m. 21 April 1987. *B.M. Boom and D. Gopaul 7567* (MO!); Holitipu, trail betw. camp and airstrip and surrounding area. 05°59′N 61°03′W. Alt. 1100 m. Tepui savanna and gallery forest. 7 February 1996. *D.H. Clarke 1037* (NY!, U!); Paruima, 5 km N, Auratoi Savanna. 05°51′N 61°05′W. Alt. 760 m. 21 July 1997. *D.H. Clarke et al. 6137* (U!); Cuyuni-Mazaruni Region. Pakaraima Mts., 12 m waterfall, large Partang River tributary, 12.7 km NE Imbaimadai. Scrub forest merging with riparian gallery forest. 5°48′N 60°14′W. Alt. 700 m, 25 May 1992. *B. Hoffman 1868 and C.L. Kelloff, G. Gharbarran, and S. Sprague* (NY!, US!); Kaieteur savanna. 1936. *G. Hollister s.n.* (NY!); Pakaraima Mts. Mt. Latipu, top (Mazaruni R.), 5°57′N 60°38′W. Alt. 900 m. 10 November 1979. *P.J.M. Maas and L.Y.T. Westra 4208* (U!); Pakaramia Mts., Mt. Aynatoi (sandstone). 5°55′N 61°W. Dry sandstone rocks near falls. 16 October 1981. *P.J.M. Maas et al. 5781* (COL!, MO!, U!); Kaieteur Plateau, 12 May 1944. *B. Maguire and D.B. Fanshawe 23419* (NY!, U!); Fleuve Oyopack, Savane roche, Roche Canari zozo, rive gauche. 8 July 1969, *R.A.A. Oldeman 332* (U!); **French Guiana**. Region de la Haute Crique Armantabo, bas Oyapock, 21 February 1981. *J.J. de Granville 165* (U!). **Brazil**. Amazonas. Rio Tuari (afluente de Rio Negro), Lago Uirauacu (=Passaro Grande em Lingua Geral), 0°20′N, 67°20′W. 13 November 1987. *M.L. Kawasaki 144* (U!, US!); Rio Uapes, Panure, catinga. 15 November 1947. *J.M. Pires 1026* (COL!, US!). Bahia. Santa Cruz Cabralia, Mata costeira. 5 November 1966. *R.P. Balem and R.S. Pinheiro 2841* (F!); Marau, resting. 18 January 1967. *R.P. Balem and R.S. Pinheiro 3180* (F!); Una-Ilheus. 39°02′W, 15°07′S, Alt. 70 m. 25 December 1975. *P. Bamps 5053* (U!); Ba. Lancois. Rio Mueugezinho, Proximo a BR-242. Em direcao a Serra Brajao. Alt. 1000 m. 20 December 1984. *A. Furlan et al. 37123* (K!). Km 10, Ponta-Olivacea road, Mpio Ilheus, 14°50′S, 39°2′W, Alt. 30–50 m. 10 February 1985. *A. Gentry and E. Zardini 50008* (MO!); Coastal Zone, 16 km S of Cumuruxatiba, 39°15′W, 17°13′S. Alt. 0–50 m. 18 January 1977. *R.M. Harley 18095* (K!, U!); Mato Grosso, margem direita de R. Juruena, morrinio da cochoeira de S. Joao da Barra. 10 June 1977. *N.A. Rosa and M.R. Santos 2081* (MO!, U!); Mun. Lencois, BR-242, 3–8 km W del desvio a Lencois. 12°28′S, 41°22′W, Alt. 880 m. 26 November 1992. *R. Mello-Silva and J. Vicente 5800* (K!, F!, MO!); Mun. Itabuna, 10 km S de Pontal (Ilheus), camino a Olivenca, local de extraccion de arena, 14°54′S, 39°02′W. Alt. 50 m. 4 December 1992. *R. Mello-Silva and J. Vicente 5583* (K!); Mpio de Castro Alves, Serra da Jiboia, 12°51′11″S, 39°29′19″W. 8 July 1992. *L.P. de Quieroz, S. Mayo, M. Nadruz, T.S.N. Sena, and M.L.S. Guedes 2946* (K!); Mun. de Una, Estrada Ilheus-Una, ±30 km au Sul de Olivenca, 15°12′S, 39°03′W. Alt. 40 m. 2 December 1981. *G.P. Lewis and A.M. de Carvalho 722* (K!); Moun. De Ilheus, Estrada Olivenca, Villa Brasil, a 7 km de Olivenca. Restinga. 13 January 1981. *A.M. Carvalho and J. Gatti 485* (K!); BA-Estrada Macuge-Andarai. 17 December 1984. *A.M. Giulietti et al. 36893* (K!); Mun. Lencois, Trilha Lencois-Capao, 12°33′34″S, 41°24′66″W. Alt. 650 m. 28 January 1997. *B. Stanard, S. Atkins, E. Saar, L. Passos, and M.L. Guedes 4581* (K!); Mun. Lencois, Morro da Chapadinha, Chapadinha, divisa com Brejoes, 12°27′00″S, 41°25′00″W. Alt. 750 m. 24 November 1994. *E. Melo et al. 1328* (K!); Olivenca km 21 para a Faz. Ipiranga ao Norte. 10 October 1972. *T.S. Santos 2456* (P!); Mun. Lencois, Chapadinha, Lencois, proximo ao Rio Mucugezinho, 12°27′44′S, 41°25′12″W. Alt. 810 m. 27 September 1994. *G. Stam, A.M. Giulietti, and H.P. Bautista 922* (K!); Mun. Lencois, Serra da Chapadinha, 12°27′41″S, 41°25′16″W. Alt. 900 m. 05 January 1996. *A.M. de Carvalho et al. 2178* (K!). Para: Maraba, Alro de Serra, arredores do N5. 12 May 1982. *A. Mesquita, R.B. Gilberto, and L. Marinho 116* (F!, K!, MO!); Sete Varas airstrip on Rio Curua, 0°95′S, 54°92′W. 6

August 1981. *J.J. Strudwick, G.L. Sobel et al. 4343* (K!); Maraba, Serra de Carajas. 12 May 1983. *N.L. Meneses s.n.* (K!); Mpio de Ameirim, reserva florestal da SEMA, 0-1°S, 52–53°W. 10 October 1987. *A.S. Tayares 117553* (MO!). Rio de Janeiro. *sine loc. V. Soares 435* (K!). Roraima. Estrada Manaus-Caracarai km 130, campina das Pedras. 25 May 1974. *W. Rodrigues, A. Loureiro, and D. Coelho 9308* (MO!). **BOLIVIA**. Santa Cruz. Vallegrande Prov., Corosito, 2 km al. S de los Sitanos. 18°52′5″S, 64°57′0. Alt. 1400 m. 2 September 1989. *I.G. Vargas 286* (F!, MO!).

Along with *Sobralia elisabethae* and *S. granitica*, it creates a group of unique species characterized by narrow leaves and white flowers with various ornamentation on the lip disk.

S. liliastrum is similar to *S. granitica*, but has larger flowers (58–62 mm long flower segments vs. 40–45 mm in *S. granitica*), the color of the lip keels (orange vs. light yellow in *S. granitica*), the raised keels (only the central keel notably raised in *S. liliastrum* vs. with two, subparallel keels at base, the disk with 9 erose-denticulate thickened keels in *S. granitica*), and the presence of pseudopollen on the *S. liliastrum* lip. The flower segments of *S. elisabethae* and *S. liliastrum* are similar in size, but they differ in lip details. In the former species, the lip is adorned with thickenings along veins running from a pair of keel-like, crenulate basal calli nearly to the apex, sometimes the thickenings are not visible in the center of the lip but distinct in its apical part anyway.

3.1.1.5. *Sobralia elisabethae* R.H. Schomb.

Verh. Vereins. Beförd. Gartenbaues Königl. Preuss. Staaten **15**: 137. 1841. Type (designated by Romero-González, 2003: 129): Venezuela. Bolivar. Vicinity of Mount Roraima, 1836, *R.H. Schomburgk 1059* (Lectotype: BM!, Isolectotypes: BM!, K!, P, W! 7463).–Baranow and Szlachetko. The taxonomic revision of the Sobralia Ruiz & Pay. (Orchidaceae) in the Guyanas (Guyana, Suriname, French Guiana). Pl Syst Evol. **302**: 338. 2016.–Szlachetko et al. Materials to the Orchid Flora of Colombia **3**: 253. 2020.

Plants 50–90 cm high, caespitose, erect, slender. Leaves numerous, up to 26 cm long and 2.5 cm wide, narrowly lanceolate, long-acuminate, suberect. Inflorescence 6–10 cm long, terminal, laxly 5–8-flowered, rachis fractiflex. Flowers opening successively, white, with yellow lip throat and keels. Floral bracts 8–40 mm long, ovate-lanceolate. Pedicel and ovary 34 mm long, slender. Dorsal sepal 50–60 mm long, 10–13 mm wide, narrowly lanceolate, acute to acuminate. Lateral sepals 55–65 mm long, 14–16 mm wide, lanceolate, subfalcate, acute. Petals 50–60 mm long, 10–13 mm wide, narrowly lanceolate, acute, subfalcate. Lip 60 mm long, 35–40 mm wide, oblong ovate in general outline, more or less notched at the apex, crenulate and undulate along margins, especially in the apical half, with thickenings along veins running from a pair of keel-like, crenulate basal calli nearly to the apex, sometimes the thickenings not visible in the center of lip but distinct in its apical part anyway. Gynostemium ca 30–40 mm long, apical wings 10–15 mm long, distinctly exceeding the column apex, linear, slightly falcate, acute (**Figure 5**).

Ecology: Terrestrial or litophytic in savannas, among rocks, xerophytic forests, and disturbed forests next to the roads. Flowering throughout the year.

Distribution: Venezuela, Ecuador, Colombia, Brazil, French Guiana, Guyana, Peru. Alt. up to 900 m.

Conservation status: EOO—LC, AOO—EN.

Representative specimens (**Supplementary Map 4**)—**Colombia**. Amazonas. Corregimiento departamental de la Pedrera. Margen izquierda del Río Caquetá, Cerro Yupati. 1°17′49″S, 69°37′03″W. Alt. 200–400 m. 6 August 1997. *D. Cardenas, C. Marin, R. Lopez, and N. Rodriguez 8528 and 8563* (COAH!); Santa Isabel, sitio sabanas de Solarte. 1°05′S, 71°10′W. 4–6 December 1996. *M.V. Arbelaez, U. Matapi, and N. Matapi 681* (COAH!); Araracuara. 3 March 1986. *P. Palacios and B. Plazas 1164* (COAH!). Caquetá. Araracuara. Orilla del Río Caquetá, balcon del Diablo. 0°36′S 72°24′W. 19 November 1993. *D. Cardenas, G. Gangi, and J. Manaidego 4135* (COAH!); Parque Nacional Natural Chiribiquete. Río Cunare, Raudal del Tubo. 0°26′N 72°30.5′W. 3 February 1992. *N. Hernandez and N.C. Penuela CHI69* (COAH!); Solano, margen izquierda del Río Caquetá, Sitio Paujil (Area del Caño Paujil), 10 km al. NO de Araracuara. 0°45′–0°48′S, 72°20′–72°25′W. Alt. 100–350 m. 10 November 1992. *V. Arbelaez and V. Hernandez 326* (COAH!); Cabaceras del Río Mesay. 1–6 Mar. 1980. *M.C. Pabón 971* (COAH!). Guainía. Trocha Nabuquen. 2°51′127″N, 65°38′339″W. Alt. 500 m. 25 February 1995. *M.P. Etter, A. Munoz, L. Baptiste, and A. Repizzo 508* (COAH!); Inrida. Resquardo indigena Almidon-Ceiba, a 4 km NE de la comunidad La Ceiba, camino a Cn Vitina. En bosquocito xerofitico transitional entre el bosque de altura y la sabana, sobre superfi. 3°39′20.3″N, 67°23′40.3″W. Alt. 80–90 m. 20 October 1998. *E. Cordillo-R. et al. 372* (MO!). Guaviare. Mpio. San José del Guaviare. Serrania La Lindosa. Bosque intervenido a orillas de la carretera. Alt. 220–250 m. 5 March 1994. *D. Cardenas and G. Trujillo 4348* (COAH!); Mpio. San José del Guaviare. Carretera de San José a Puerto Arturo, km 3, alrededores de la finca Santa Gertrudis, zona de afloramientos rocosos, 02°28′20″N, 72°41′30″W. Alt. 280 m. 21 January 1996. *R. Lopez, D. Giraldo C., and H. Salgado 952* (COAH!); Mpio. San José del Guaviare. En immediaciones de Ciudad de Piedra, Serrania La Lindosa, carretera San José-El Caprichio, 02°28′28″N, 72°41′48″W. Alt. 290 m. 19 November 1995. *R. Lopez, D. Giraldo C., and H. Salgado 829* (COAH!). Meta. Mpio. La Macarena. Serrania de La Macarena, Caño Canoas, cercanias a los chorros, formaciones de roca desnuda del Escudo Guayanes. 2°28′–29′N, 70°44′W. Alt. 255–280 m. 31 December 2005. *J. Betancur, J. Aguirre, J. Contreras, and M. Rodriguez 11993* (COL!). Vaupés. Mitú & vicinity, along Río Vaupés between Río Ti and Rapids of Mandi, 23 September 1976. *J.L. Zarucchi 2115* (K!); Mpio Mitú. Camino entre la comunidad

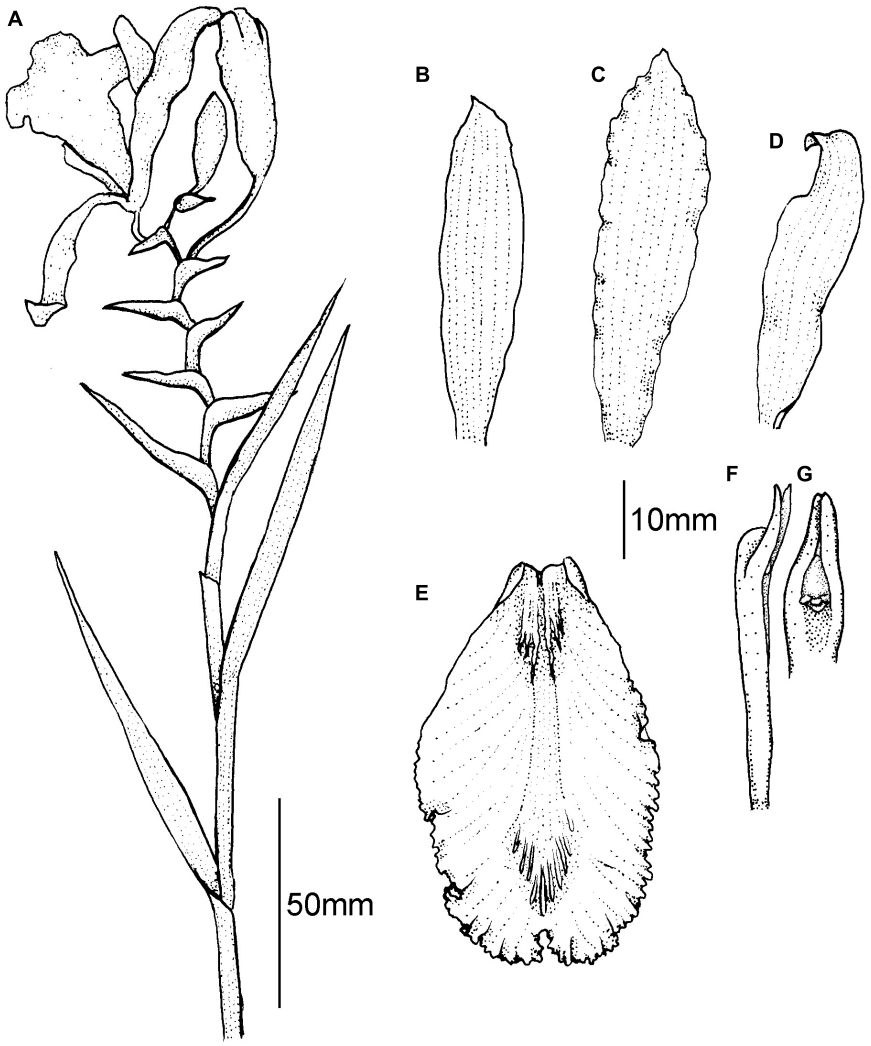

FIGURE 5
Sobralia elisabethae R. H. Schomb. **(A)** apical part of stem with inflorescence, **(B)** dorsal sepal, **(C)** petal, **(D)** lateral sepal, **(E)** lip, **(F)** gynostemium, side view, and **(G)** apical part of gynostemium, front view [drawn by P. Baranow, panel **(A)** from lectotype, **(B–G)** redrawn from Romero-González (2003)].

Mitú Cachivera y el cerro Guacamaya, 1°11′40″N, 70°14′24″W. Alt. 180–370 m. 24 September 2007. *D. Cardenas, Z. Cordero, N. Salinas, and A. Zuluaga 21087* (COAH!); Mpio. Mitú. Comunidad de Monford, *via* Monford-Mitú km 4. Sabaneta varillal a catinga de 8–10 m de altura, 0°37′17″N, 69°44′56.4″W. Alt. 160–170 m. 30 September 2007. *D. Cardenas, Z. Cordero, N. Salinas, and A. Zuluaga 21334* (COAH!); Mpio. Mitú. Sector compredito entre el cerro Guacamaya y Caño Sangre. 1°12′N, 70° 14′W. Alt. 200–300 m. June 2008. *N.R. Salinas and L.F. Jaramillo 718* (COAH!); Serrania de Taraira, 6 km al. N-W del raudal de la Libertad, Coord. 0°58′S, 69°45′W. Alt. 250 m. 27 July 1993. *R. and J. Rodrigues 609* (COAH!); Mpio. Mitú. Cabeceras de Caño Cuduyari, comunidad de Wacuraba, margen derecha del cano. Camino que conduce de la comunidad a la sabana de Yapoboda. 1°22′23″N 70°54′30″W. Alt. 200–400 m. 16 May 2006. *D. Cardenas, R. Pena, and A. Rivera 18723* (COAH!); Corregimiento departamental de Yavarate, comunidad de Bogotá-Cachivera, camina a Acaricuara. 0°49′45.3″N, 70°03′50.6°W. *N. Castano, N. Salinas, A. Zuluaga, and W. Estrada 2737* (COAH!). **Venezuela**. Amazonas. Atabapo, Cerro Huachamacari, E slope. 3°49′N, 65°42′W. Alt. 600–700 m. 3 November 1988. *R.L. Liesner 25736* (U!); Santa Lucia, Pedra de Cucui. 28 October 1967. *Farney et al. 1822* (K!); Base occidental del Cerro Yapacana, 3°38′N 66°52′W. Alt. 100 m. 10 December 1978. *O. Huber and Tillett 3023* (K!); Rios Pacimoni–Yatua, Casiquiare, 26 September 1957. *B. Maguire et al. 41583* (K!); Bolivar. Vicinity of Mount Roraima, 1836. *R.H. Schomburgk 1059* (BM!, K!, P!, W-R!, W-R!–drawing); Atabapo. Falda del extremo norte del Cerro Duida. 3°40′N 65°45′W. Alt. 800–900 m. 6 February 1982.

J.A. Steyermark et al. 126106 (BM!, K). **Guyana**. Utshi R. trail to Santa Elena, Venezuela, 05°39′N 61°09′W. Alt. 980 m. 31 January 1996. *D.H. Clarke 942* (NY!, U!); Cuyuni-Mazaruni Mts. Karowrieng River, 0.5–1 km SE Maipuri Falls, trail to rock drawings, 5°40′N, 60°13′W, Alt. 625–650 m, 15 October 1992. *B. Hoffman 3021 with T. Henkel and H. Kennedy* (NY!); 3 km SE of S end of Haiamatipu, above Kobadoi Savanna, 5°27′N 60°39′W, 549–610 m. 16 June 1991. *T. McDowell et al. 4619* (NY!, U!). **French Guiana**. Cochoeira das Arraras, esatingas entre rio Vaupes e Arary. 3 November 1945. *R. Lemos Froea 21310* (K!, US!); **Brazil**. Amapa. Rio Araguari, downriver from Porto Platon. 21 September 1961. *J.M. Pires, Wm. Rodrigues, and G.C. Irvine 51146* (U!). Ad flumina Casiquari, Kasiva et Pacimoni. 1853-4. *R. Spruce 3014* (BM!). Amazonas: Amza camp N5, 6°4′S, 50°08′W, Alt. 700–750 m. 12 May 1982. *C.R. Sperling, R.S. Secco, M. Condon, A.L. Mesquita, B.G.S. Ribeiro, and L.R. Marinho 5609* (K!, MO!); Maraba, Alto de Serra, arredores do N5, solo de canga (ferro). 12 May 1982. *R. S. Secco et al. 116* (MO!); Marraba, Serra dos Canajas. 2 April 1977. *M.G. Silva and R. Bahia 2991* (K!); Rio Negro, near mouth of Rio Xie, Vista Alegre, opposite Sao Marcelino, 0°55′N, 67°13′W. 21 October 1987. *P.J.M. Maas, D.W. Stevenson, C. Farney, J.F. Ramos, and R.P. Lima 6832* (U!).

The species is very similar to *Sobralia granitica* in flower structure. They can be distinguished by the size of the flowers—flowers of the latter species are smaller (40–45 vs. 50–65 mm long in *S. elisabethae*). Additional differences can be observed in flower color—*S. elisabethae* has a white lip with a yellow throat and reddish orange keels, while *S. granitica* has a hyaline white lip with pale yellow, elevated keels.

3.1.1.6. *Sobralia granitica* G.A. Romero & Carnevali

Harvard Pap. Bot. **5** (1): 184. 2000. Type: Venezuela. Amazonas. Municipio Atabapo, Caño Ucata, Cerro Lombriz, 9 December 1994, *G.A. Romero and S. Llamozas 3016* (Holotype: VEN; Isotypes: AMES!, K!, SEL).—Szlachetko et al. Materials to the Orchid Flora of Colombia **3**: 254. 2020.

Stems caespitose, cane-like, up to 130 cm high, terete, erect, basal internodes up to 15 cm long, leafless, apical internodes up to 2 cm long, leafy. Leaves 12 cm long, 1.5 cm wide, narrowly lanceolate, long-acuminate, rigidly coriaceous, articulate with their sheaths, the sheaths 3 cm long, tightly clasping the stem. Inflorescence terminal, sessile, elongating with age, fractiflex, successively single-flowered, subtended by a foliaceous, articulate bract, up to 5 cm long, not including the sheath. Flowers showy, with submembranaceous, widely spreading perianth segments, lasting only 1 day, sepals white, the tips greenish–yellow petals and lip hyaline white, disk of lip light yellow. Floral bracts non-articulate, up to 17 mm long, subimbricating lanceolate, long-acuminate. Pedicellate ovary up to 18 mm long. Dorsal sepal up to 40 mm long and 8 mm wide, narrowly elliptic to linear-elliptic, acute, with a short apiculus. Lateral sepals 42 mm long, 9 mm wide, narrowly elliptic to linear-oblanceolate, acute, with short apiculus, somewhat oblique. Petals up to 40 mm long and 13 mm wide, obovate-lanceolate, acute, oblique, margins of apical half undulate. Lip up to 45 mm long and 33 mm wide, oblong obovate to pyriform in outline, apically rounded, emarginate, margins above basal third undulate-crispate, the base with two, 5 mm long, subparallel keels, basally in close proximity, forming a small cavity beneath, the disk with 9 erose-denticulate thickened keels, dilated at the apex, the central five subtriangular. Gynostemium up to 30 mm long, semiterete, slender, somewhat clavate, with a pair of lateral, falcate, acute stelidia at the apex, much exceeding the anther apex, up to 6 mm long, anther white, pollinia white yellow (**Figure 6**).

Ecology: Litophytic or terrestrial on granitic outcrops and edges of white-sand shrubland. Flowering in February, March, November, and December.

Distribution: Colombia, Venezuela. Alt. 90–350 m.

Conservation status: EOO—LC, AOO—EN.

Representative specimens (**Supplementary Map 5**)—**Colombia**. Guainía. Mpio. Pto Inírida. Comunidad El Remanso. Cerro de Mavicure. Formaciones vegetales sobre roca gramitica, 3°27′N 67°58′W. Alt. 300 m. 25 March 1998. *A. Rudas, A. Prieto, D. Angel, C. Cardenas, and M. Celis 7336* (COAH!, MO!). Guaviare. PNN Nukak, San José del Guaviare, Inspec. del Tomachipan, Río Inrida, Caño Cocui, Cerro Cocui, Sabaneta on Roca, 2°08′11.8″N, 71°09′41.2″W. Alt. 350 m. 11 February 1996. *M.P. Cordoba, A. Etter, and H. Mendoza 2191* (COAH!); Mpio. San José del Guaviare. Vereda la Pizarra, Camino la Lindosa-La Recebera. December 2005. *V. Pinoz and D. Cardona 438* (COAH. **Venezuela**. Amazonas. Mpio. Atures. Bosque-laja en Cerro "Uchonhua" (lengua Piaroa), a unos 5 km al N del caserio San Pedro de Catanipo, a unos 60 km al SE de Puerto Ayacucho. 5°41′N, 67°11′W. Alt. 120–150 m. 9 November 1980. *F. Guanchez 366* (TFAV, VEN); Cerro de afloramiento granitico a 3 km al N del Cesario Piaroa "Bablilla de Pintado," al S de Puerto Ayacucho. 5°32′N, 67°31′. Alt. 90–110 m. 26 March 1981. *F. Gunachez 953* (TFAV, VEN); Cerro granitico al El del Raudal Gavilan. 5°37′N, 67°22′W. Alt. 100 m. 1 February 1991. *G.A. Romero, C. Gomez, and E. Melgueiro 2291* (AMES!, TFAV, VEN); Mpio. Atabapo. Caño Ucato, Cerro Lombiz. 9 December 1994. *G.A. Romero and S. Llamozas 3016* (VEN, AMES!, K, SEL). Bolivar. Cerro San Boja. Alt. 100–300 m. 12 December 1955. *J.J. Wurdack and J.V. Monachino 39809* (AMES!, NY, VEN).

Sobralia granitica is similar to *S. liliastrum*, but it differs in the smaller size of the flowers, (floral segments length of *S. granitica* is 40–45 mm while in *S. liliastrum* 58–62 mm), the color of the keels (light yellow in *S. granitica* vs. orange in *S. liliastrum*), the raised keels (vs. only the central keel notably raised in *S. liliastrum*), and the absence of pseudopollen on the lip (vs. present in *S. liliastrum*). The plants are easily distinguishable in the field, but only with careful examination

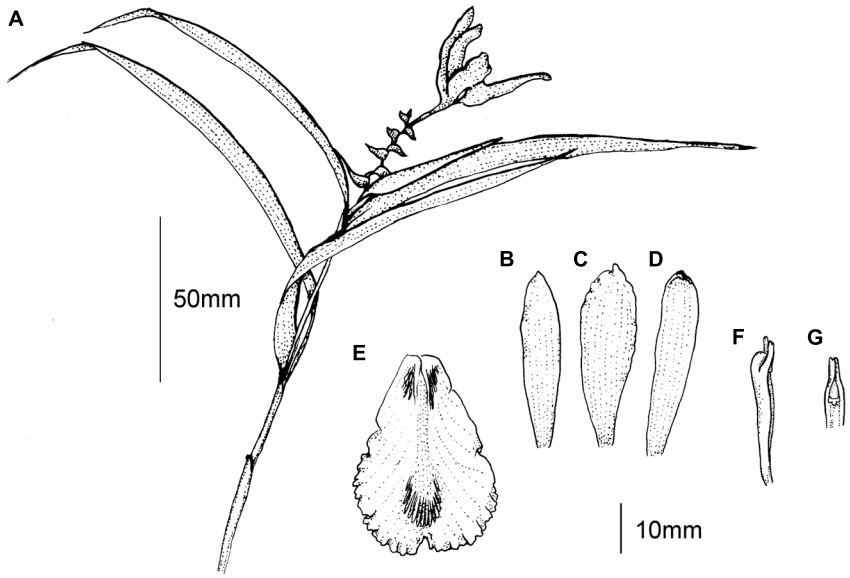

FIGURE 6
Sobralia granitica G. A. Romero & Carnevali **(A)** apical part of stem with inflorescence, **(B)** dorsal sepal, **(C)** petal, **(D)** lateral sepal, **(E)** lip, **(F)** gynostemium, side view, and **(G)** apical part of gynostemium, front view [drawn by P. Baranow, **(A)** from *Rudas* et al. *7336*, **(B–G)** redrawn from Romero-González (2003)].

of the keels, they can be told apart in herbarium material (Romero-González, 2003).

3.1.1.7. *Sobralia luerorum* Dodson

Orquideología **21** (1): 33. 1998. Type: ECUADOR. Azuay. Cuenca to Guarumales, between dam and casa de Maquinas, Alt. 1500 m. 9 March 1985. *C.H. Dodson, P. Dodson, C., and J. Luer 15872* (Holotype: RPSC!; Isotypes: AMES, QCA, QCNE–illustration of type).—Szlachetko et al. Materials to the Orchid Flora of Colombia **3**: 255. 2020.

Plants up to 350 cm tall, robust, caespitose, stem cane-like, surrounded for the basal portion with clasping sheaths. Leaves up to 35 cm long, 10 cm wide, elliptic to elliptic-lanceolate, coriaceous, acuminate, distichous, plicate, heavily veined on the underside, clasping the stem at the base, articulated to leaf-sheath surrounding the stem. Inflorescence up to 20 cm long, fractiflex, with a large, spathe-like bract at each node, the flowers produced singly in succession over prolonged periods with flowering concurrent throughout the population. Sepals and petals white, the lip white heavily splashed with red–purple on the lamina and in the throat, veins in the lip yellow. Dorsal sepal up to 75 mm long and 20 mm wide, narrowly oblong, acute. Lateral sepals up to 70 mm long and 22 mm wide, narrowly elliptic, oblique, and acute. Petals up to 70 mm long and 22 mm wide, oblong-obovate, obtuse, apical margins more or less erose. Lip up to 70 mm long and 40 mm wide, elliptic, retuse at the apex, with a pair of shallow lamellae in the throat, margins entire or inconspicuously dentate-erose, undulate. Gynostemium 26–30 mm long, slender at the base, expanded on each side toward the apex to form falcate horn-like apical stelidia (**Figure 7**).

Ecology: Epiphytic or terrestrial on road cuts and embankments. Flowering in January–April, August, and November.

Distribution: Colombia, Ecuador. Alt. 1500–2200 m.

Conservation status: EOO—LC, AOO—EN.

Representative specimens (**Supplementary Map 6**)—**Ecuador**. Azuay. Cola de San Pablo, Noreste de Paute en el Río Paute, Entre Guarumales y el tunel. Alt. 1500 m. 9 March 1985. *C. and P. Dodson, C. and J. Luer and A. Hirtz 15782* (AMES!, RPSC!); Quebrada Chorro Blanco, Río Paute Valley, 8 km SE of the Paute Dam at Amaluza, 78°33′W. 2°38′S. Alt. 1700 m. 3 February 1988. *U. Molau, B. Eriksen, and M. Fredrikson 2882* (MO!). Napo. Km 117–134, Quito-Tena, beyond Cosanga at Cordillera de Guacamayo. Alt. 1900–2100 m. 17 January 1990. *C.H. Dodson and T. Neudecker 19193A* (MO *ex* RPSC!). Tungurahua. Baños–Puyo road near Río Negro, border with Santiago-Zamora. Alt. 1200 m. 24 April 1980. *A. Gentry and C. Bonifaz 28740* (MO!). **Colombia**. Antioquia. Mpio Briceno. Vereda San Fermin, 2–3 km sobre la *via* Ventanas (Mpio Yaruma) Briceno, 7°10′N, 75°30′W. Alt. 1700–1900 m. 3 November 1990. *R. Callejas and M.V. Arbelaez 9603* (AMES!, NY!). Putumayo. Valle de Sibundoy, 1 km S Balsayaco. Alt. 2200 m. 20 August 1963. *M.L. Bristol 1319* (AMES!).

Sobralia luerorum is similar to *S. gloriosa*, but can be distinguished by the larger flowers of thinner texture, white sepals and petals, the lip with red–purple splashing

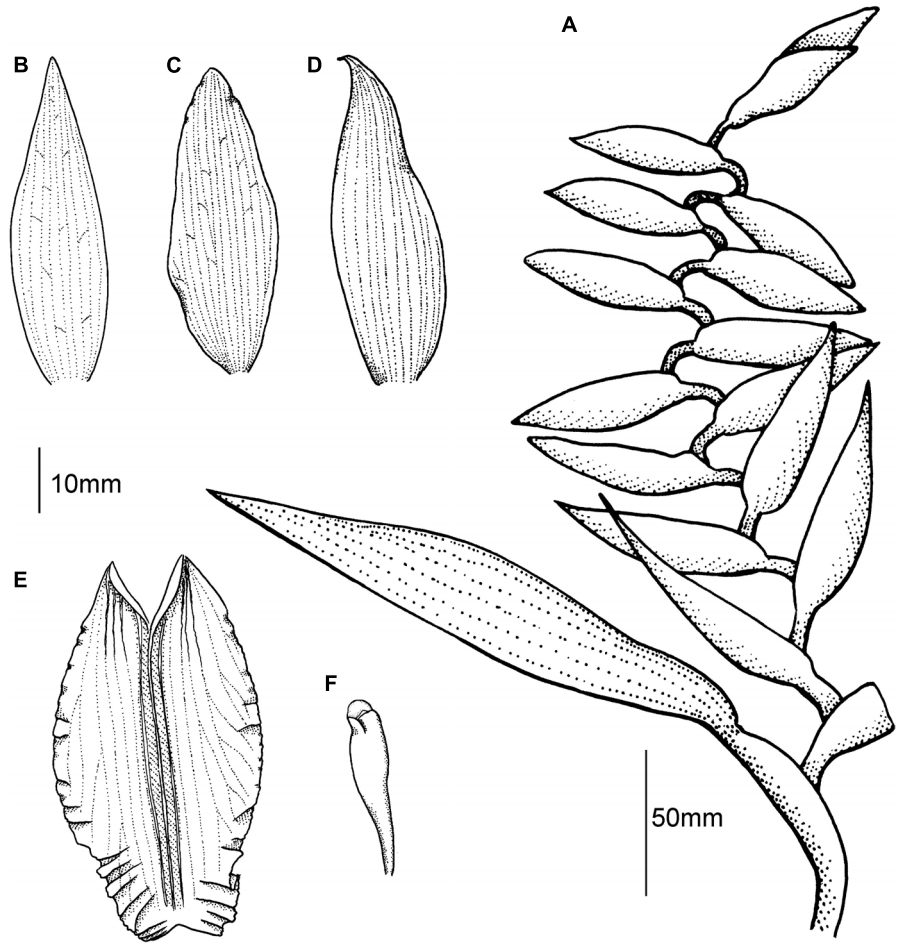

FIGURE 7
Sobralia luerorum Dodson **(A)** apical part of stem with inflorescence, **(B)** dorsal sepal, **(C)** petal, **(D)** lateral sepal, **(E)** lip, and **(F)** gynostemium, side view (drawn by P. Baranow from *Bristol 1319*).

on the lamina and throat (in *S. gloriosa* sepals are yellow to brown, the petals are white to yellow and lip is white with purple striation), the elliptic-retuse lip (vs. broadly elliptic and bilobed at the apex lip in *S. gloriosa*), and a pair of calli in the throat of the lip inconspicuous (rather than large and conspicuous, as in *S. gloriosa*).

According to the protologue, the specimens of *S. luerorum* reach up to 200 cm in height. However, some of the examined herbarium collections (e.g., *C. and P. Dodson, C. and J. Luer and A. Hirtz 15782)* and the plants cultivated in our living collection allow us to verify the information and state, that the stems can reach up to 350 cm.

3.1.1.8. *Sobralia gloriosa* Rchb.f.

Xenia Orchid. **2**: 178. 1873. Type (designated by Garay, 1978: 122): Ecuador. Pichincha. From the forest of the Western side of Pichnincha, Alt. 2300 m. Sep. *W. Jameson 32* (Lectotype: W! 21547).–Garay in Harling and Sparre. Fl. Ecuador. Orchid. **9**: 123. 1978.—Szlachetko et al. Materials to the Orchid Flora of Colombia **3**: 256. 2020.

Plants over 200 cm tall. Leaves up to 37 cm long and 12 cm wide, ovate to ovate-elliptic, long-acuminate. Inflorescence up to 30 cm long, rachis fractiflex, loosely many-flowered. Floral bracts up to 80 mm long, cymbiform, the lowermost ovate-lanceolate, acute, the upper ones obtuse, longer than the ovary. Pedicellate ovary up to 22 mm long, cylindrical, glabrous. Flowers produced in succession, rather fleshy, creamy white with purple striation on lip. Dorsal sepal up to 60 mm long and 15 mm wide, ovate-lanceolate to oblong lanceolate, acute, dorsally mucronate, the margins involute and undulate. Lateral sepals up to 50 mm long and 18 mm wide, connate for 5 mm basally, obliquely ovate-lanceolate to oblong lanceolate, acute, mucronate dorsally, with involute and undulate margins. Petals up to 60 mm long and 20 mm wide, oblong obovate to narrowly elliptic, obtuse, with crenulate and undulate margins above. Lip up to 50 mm long and 40 mm wide, rhombic-elliptic in general outline, obscurely 3-lobed, lateral lobes erect, enfolding the

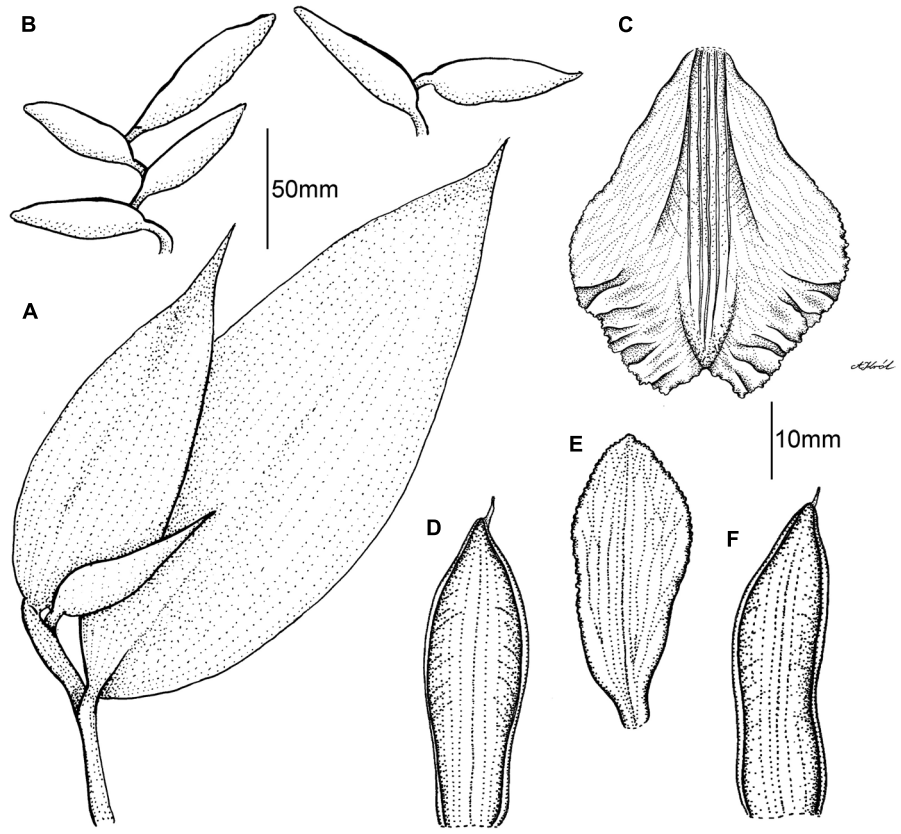

FIGURE 8
Sobralia gloriosa Rchb. f. **(A)** apical part of stem, **(B)** fragments of the inflorescence, **(C)** lip, **(D)** dorsal sepal, **(E)** petal, and **(F)** lateral sepal [drawn by P. Baranow **(A,B)** and A. Król **(C–F)** from the lectotype].

column, rounded at apex; middle lobe suborbicular in outline, bilobed apically with wavy-crispate margin; disk obliquely bicallose at the base and with 5–7 parallel verrucose thickened veins from the base to the apex. Gynostemium up to 40 mm long, clavate, stelidia do not exceed the anther apex (**Figure 8**).

Ecology: Terrestrial on steep embankments in wet montane cloud forest. Flowering throughout the year.

Distribution: Ecuador, Colombia, Peru (Garay, 1978). Alt. 1800–2300 m.

Conservation status: EOO—VU, AOO—EN.

Representative specimens (**Supplementary Map 7**)—**Ecuador**. Carchi. Trail from Rafael Quindis mountain finca to Río Verde and short distance up Río Verde, 0°52′N, 78°8′W. Alt. 1890 m. 28 November 1987. *W.S. Hoover and S. Wormley 1873* (MO!); Ridge to NE of Rafael Quindis mountain finca, 0°52′N, 78°8′W. Alt. 2000 m. 29 November 1987. *W.S. Hoover 2024* (MO!); Trail from Rafael Quindis mtn finca to Río Verde and short distance up Río Verde, 0°52′N, 78°8′W. Alt. 1890 m. 28 November 1987. *W.S. Hoover and S. Wormley 1872* (MO!). Imbabura. 8 km east of Lita on road to Ibarra and 8 km up road from Cachaco to Santa Rosa de Cachaco to an elev 1150 m and hiked up ridgeline to 1550 m alt. 19 January 1987. *C.H. Dodson, A. Hirtz, D. Benzing C., and J. Luer 16886* (RPSC!). Pastaza. On roadside at km 70 Baños to Puyo. Alt. 1900 m. 18 February 1963. *L.B. Thien 2270* (F!). Pichincha. km 88–92, Quito-Sto Domingo. Alt. 1200 m. 4 July 1979. *C.H. Dodson, M. Fallen and P. Morgan 7776* (RPSC!); Road from Quito to Santo Domingo *via* Chiriboga, 8 December 1986. *C.H. Dodson and E. Hagsater 16702* (RPSC!); Reserva Floristica-Ecologica Río Guajalito, km 59 de la carretera antiqua Quito-Sto Domingo de Los Colorados, a 3.5 km al NE de la carretera, estribaciones occidental del Volcan Pichincha. 0°13′53″S, 78°48′10″W. Alt. 1800–2200 m. 28 December 1985. *J. Jaramillo 8312* (MO!); Chiriboga road, old Santo Domingo-Quito road, 31 km northeast of Alluriquin, Alt. 6000 ft. 5 August 1980. *R.P. Sauleda et al. 4000* (AMES!); along road Nanegal-Nanegalito. Alt. 1200–1500 m. 9 July 1991. *H. van der Werff, B. Gray, and G. Tipas 12264* (MO!). **Colombia**. Valle del Cauca. Along road between San José del Palmar and Ansermanuevo. 4°49′N, 76°09′W. Alt. 1960 m. 12 May 1983. *T.B. Croat 56717* (COL!, MO!, NY!); Mpio. El Cairo. Vereda El Pacifico, 10 km desde el desvio a San José del Palmar de la carretera Albán-Cartago.

4°48′42″N, 76°10′16″W. Alt. 1867 m. 29 December 2007. *R. Arevalo, J. Betancur, N. Salinas, L. Clavijo, and A. Zaluaga 804* (COL!); Hacienda Tokio, behind microwave tower, ca 10 km S of Queremal, 3°30′N, 76°42′W. Alt. 2000 m. 26 February 1983. *A. Gentry, A. Juncosa, and F. Gomez 40817* (COL!, MO!). Valle/Choco: Mpio El Cairo, Correg. Boqueron, Vereda Las Amarillas, Serrania de Los Paraguas, along road to and beyond Cerro del Ingles, 17–23 km W of El Cairo, 4°45′N, 76°20′W, Alt. 1750–2050 m. 13 May 1988. *J. L. Luteyn, P. Silverstone-Sopkin, M. Dolores Hereida, and N. Paz 12274* (AMES!). Valle: Alt. 2000 m. May 1939. *E. Dryander 2359* (US!).

Along with *Sobralia luerorum*, *S. gloriosa* has strongly fractiflex rachis which allows to separate the two species from the other representatives of the section with broad leaves (over 5 cm width). The taxa can be distinguished by flower color and lip protuberances. The differences between them are indicated in the notes concerning *S. luerorum*.

3.1.1.9. *Sobralia ruckeri* Linden & Rchb.f.

Bonplandia (Corrientes) **2**: 278. 1854. Type: Colombia. *Sine prec. loc. L.J. Schlim 1203* (Holotype: W!, UGDA-DLSz!–drawing).—Garay & Dunsterville. Venezuelan Orchids Illustrated 406. 1959.—Szlachetko et al. Materials to the Orchid Flora of Colombia, **3**: 257. 2020.

= *Sobralia charlesworthii* hort., Gard. Chron. 353. 1910. Type: cult. ex *Charlesworth* (Holotype: K! 000364502).

Plants up to 300 cm tall, robust, stem up to 1.5 cm in diameter, erect, leafy, growing in dense clumps, slightly compressed or subterete. Leaves up to 35 cm long and 12 cm wide, lanceolate to ovate-lanceolate, attenuate, the uppermost leaves tend to be somewhat cymbiform in the basal part, sheaths spacious, ribbed. Inflorescence up to 6-flowered, several of which can be out simultaneously; strongly sinuous and stout, subterete. Floral bracts basally cymbiform and in their apical portion almost identical to the leaves but smaller—up to 200 mm long, getting progressively smaller toward the apex of raceme. Pedicellate ovary varies in length from 30 mm in apical flowers up to 100 mm in the basal ones. Sepals very dark magenta–purple, sepals fairly intense rose–purple with a pale mid-vein, petals rose–purple with a pale mid-vein, lip dorsally rose–purple grading to a very dark wine–purple at the apex, ventrally with a large patch of light rose–purple at the base, changing abruptly to very dark wine–purple for the remainder. Dorsal sepal up to 85 mm long and 25 mm wide, ligulate-lanceolate to linear-oblanceolate, acute, mucronate, fleshy, basally connate with lateral sepals. Lateral sepals up to 85 mm long and 20 mm wide, oblanceolate, acute, mucronate, more or less falcate. Petals up to 85 mm long and 33 mm wide, widely oblanceolate or oblong obovate, oblique, firm but much thinner than the sepals, the thin margin of the apical third variably undulate. Lip 80 mm long, 60 mm wide, ovate to elliptic, axis strongly thickened ventrally into a yellow ridge that starts from the thick, finely sulcate, transverse thickenings that prevent the spreading of the base of the lip, the axial ridge lightly grooved, rather soft, rugulose, in apex finely bullate, white. Gynostemium ca 35 mm long. Stelidia linear, acute, slightly exceeding the gynostemium apex (**Figure 9**).

Ecology: Terrestrial at the edges of forests and clearings. Flowering from February till April and in September.

Distribution: Colombia, Venezuela. Alt. 1200–2400 m.

Conservation status: EOO—LC, AOO—EN.Representative specimens (**Supplementary Map 8**)—**Colombia**. Antioquia. Region de Murri, road between Nutibara and La Blanquita, 14.3–17.5 km from centro of Nutibara, 6°45′N, 76°23′N, 1620–1860, 10 February 1989. *J.M. Mc Dougal, D. Restrepo, and D.S. Sylva 3853* (MO!); Mpio. Frontino. Corregimiento Nutibara, Cuenca alta del Río Cuevas. Alt. 1640 m. 11 April 1987. *D. Sanchez et al. 1048* (MO!); Mpio. Frontino. Corregimiento Nutibara, Cuenca alta del Río Cuevas. Sobre tulud, 2 m de alto. Alt. 1640 m. 11 April 1987. *F.J. Roldan, J. Betancur et al. 1048* (COL!, NY!); Mpio. Frontino. Corregimiento Nutibara, Cuenca alta del Río Cuevas. Alt. 1750 m. 14 April 1987. *D. Sanchez et al. 1139* (COL!, MO!, NY!). Boyacá. Mpio. Duitama. Trayecto entre la vereda El Carmen y Virolin. 21 September 1994. *J.L. Fernandez-Alonso, C. Ariza, A. Baena, J. Gomez, A. Espinoza, A. Pico, D. Riano, and D. Sarmiento 12070* (COL!); Carretera Duitama. Charala, 65 km de Duitama. Adelante de Virolin. 9 June 1972. *G. Lozano C. 2228* (COL!). Norte de Santander. Ocaña. Alt. 1700–2000 m. *L.J. Schlim 1203* (W!). Putumayo. Entre el Pepino y Mocoa. Cerca al Río Putumayo. Alt. 1200 m. 11 January 1963. *A. Fernandez P. 6015* (COL!). Santander. Gambita. Alt. 2400 m. 12 February 2010. *M. Ospina H. 1611* (COL!). Valle del Cauca. Km 18 y km 20 de la carretera de Cali a Buenaventura entrado por la finca Zingara. Cumbre de la Cordillera occidental. Alt. 1500–2000 m. 28 February 1988. *I. Cabrera R. and H. van der Werff 15766* (MO!). **Venezuela**. Zulia. Sierra de Perijá, Loma arriba de la quebrada del Río Omira-kuna (Tumuriasa), cerca de la frontera Colombo-Venezolana suroeste de Pishikakao e Iria hacia la Mision de Sucurpo. Alt. 1980 m. 27 March 1972. *J.A. Steyermark, G.C.K., and E. Dunsterville 105664* (AMES!).

The characteristic leaves which are gradually getting smaller toward the apical part of the stem and fluently transform into leaf-like floral bracts are unique among the whole genus and allow us to distinguish the species at the first glance. The features, along with the shape and color of the floral segments, especially the lip, prompted the decision to synonymize *S. charlesworthii* under the name *S. ruckeri*. Such a concept was mentioned in the description of *S. charlesworthii*—it suggests that *S. charlesworthii* may be just a form of *S. ruckeri*.

The only species that could be misidentified with *Sobralia ruckeri* is *S. splendida*, but the latter taxon differs in the protuberances present on the lip surface. The details are listed in the notes concerning *S. splendida*.

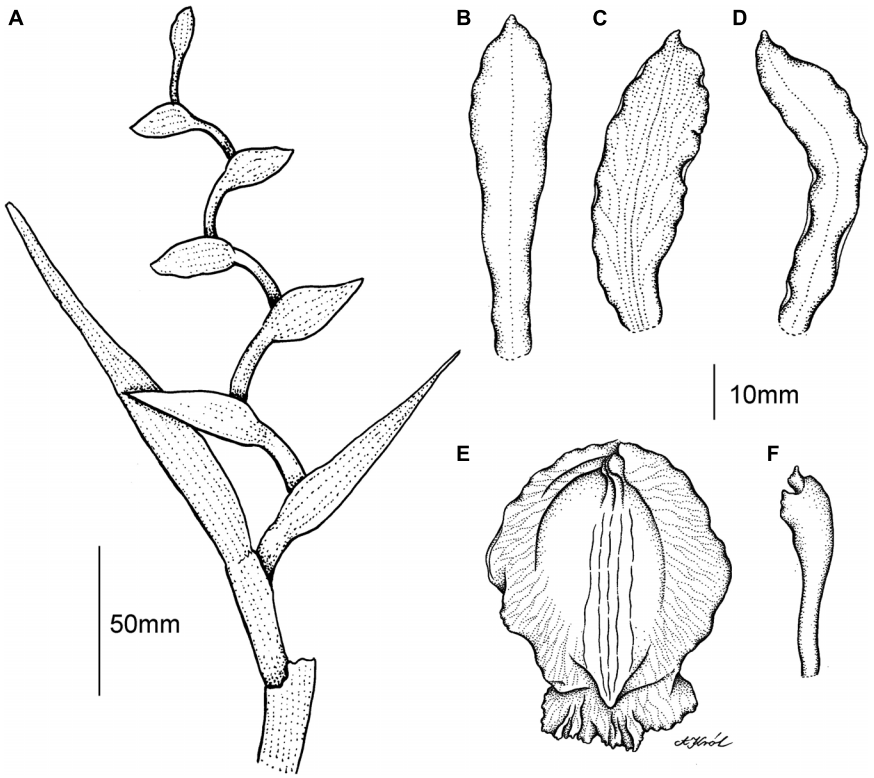

FIGURE 9
Sobralia ruckeri Linden & Rchb. f. **(A)** apical part of stem with inflorescence, **(B)** dorsal sepal, **(C)** petal, **(D)** lateral sepal, **(E)** lip, and **(F)** gynostemium, side view [drawn by P. Baranow **(A)** and A. Król **(B–F)** from the holotype].

3.1.1.10. *Sobralia gambitana* Baranow, Szlach. & Kindlmann, *sp. nov.*

Type: COLOMBIA. Santander. Mnio Gámbita, vereda El Palmar. Alt. 2500 m. 12 May 1982. *A. Becerra and M. Constanza 23* (Holotype: COL! 256896, UGDA-DLSz!–drawing).

Similar to S. *tamboana in habit and flower structure. However, it can be separated by the pair of thickenings at the base of lip—they are fused together in the new species while in* S. tamboana, *they are separated. The two species differ also in the color of flowers—they are lilac with purple lip edges and yellow at the center in the new entity. In S.* tamboana, *flowers are pale yellow with a red-brown wash inside the throat of the lip and a red-brown spot on the lamina of the lip. Moreover, S. gambitana is two times as tall as S. tamboana (ca 250 cm vs. 120 cm) while its floral segments are distinctly smaller than those of S. tamboana (60–66 vs. 78–92 mm).*

Etymology: Named in allusion to Colombian Municipio Gámbita, where the type material was collected.

Plants ca 250 cm tall. Leaves 13–14 cm long, 1.8–2.5 cm wide, oblong elliptic linear-lanceolate, acuminate, basally cuneate, strongly plicate. Inflorescence ca. 4 cm long, rachis flexouose, glabrous. Flowers lilac, lip with purple edges and a yellow line in the center. Floral bracts up to 20 mm long, becoming smaller toward the rachis apex, lanceolate-cymbiform, acuminate. Ovary 10–11 mm long, cylindrical. Dorsal sepal 66 mm long, 22 mm wide, lanceolate or elliptic-lanceolate, shortly acuminate. Lateral sepals 60 mm long, 20 mm wide, oblong elliptic, shortly acuminate, inconspicuously oblique. Petals 62 mm long, 36 mm wide, obovate, linear and falcate basally, acute, margins slightly crenate. Lip 60 mm long, 50 mm wide, rhombic-elliptic in outline, deeply concave basally, margins of apical half undulate, base with two united keels running up to one-third of the lip. Gynostemium 37 mm long, slightly falcate, club-like, apical stelidia triangular, falcate, acute, not exceeding the anther (**Figure 10**).

Ecology: No data. Flowering in May.

Distribution: Colombia (Santander). Alt. 2500 m.

Conservation status: EOO—CR, AOO—CR.

Representative specimens (**Supplementary Map 9**)–**Colombia**. Santander. Mnio Gámbita, vereda El Palmar. Alt. 2500 m. 12 May 1982. *A. Becerra and M. Constanza 23* (COL! 256896, UGDA-DLSz!–drawing).

The descriptions of the new taxon on the basis of a single collection may be doubtful, but in this case, we have the combination of the morphological features and flower color that convince us that the collection deserves the status of a separate species.

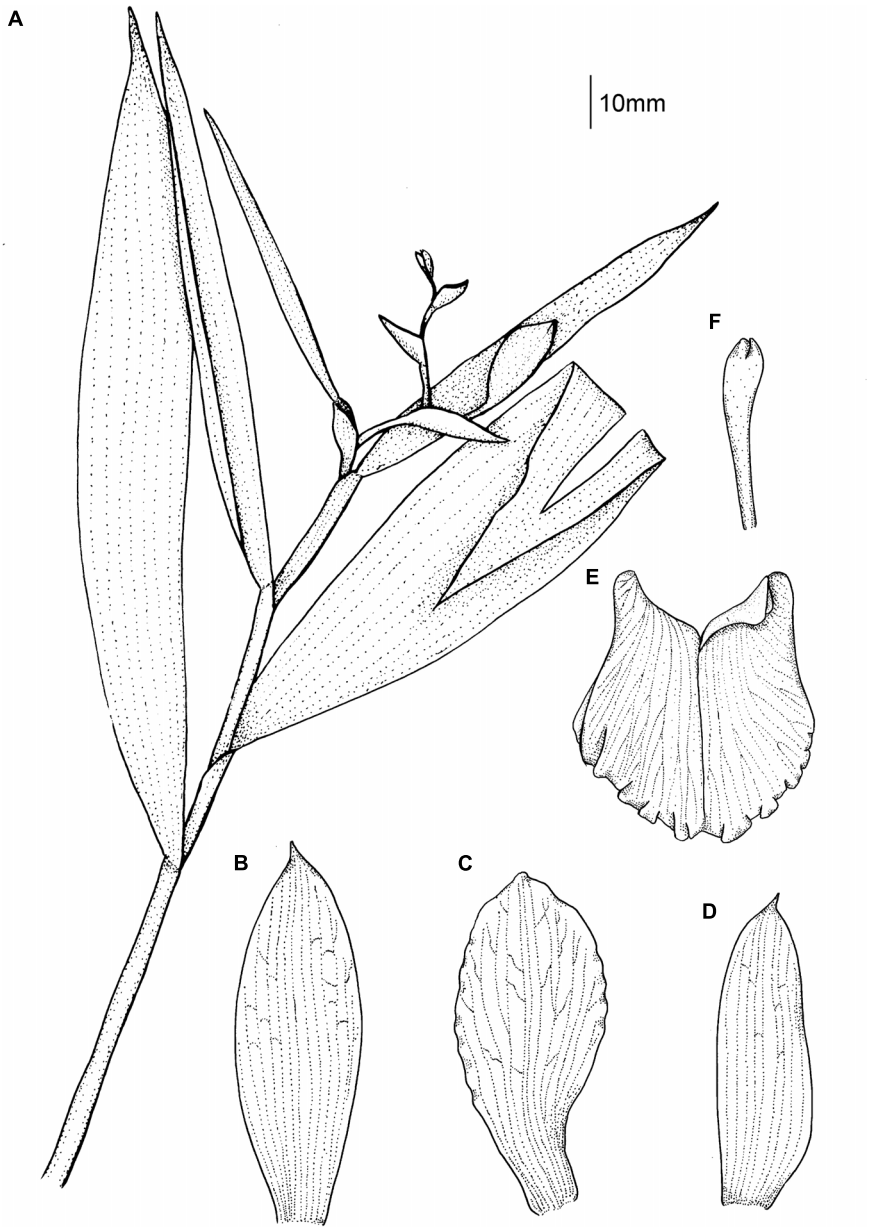

FIGURE 10
Sobralia gambitana Baranow, Szlach. & Kindlmann **(A)** apical part of stem with inflorescence, **(B)** dorsal sepal, **(C)** petal, **(D)** lateral sepal, **(E)** lip, and **(F)** gynostemium, side view (drawn by P. Baranow from the holotype).

Sobralia gambitana is similar to *S. tamboana* in habit and flower structure. However, it can be separated by the position of the lip basal thickenings. In the new species, the two basal ridges are fused together while in *S. tamboana* they are separated. The two species differ also in flower color—the new species are lilac with purple lip edges and yellow at the center. In *S. tamboana* flowers are pale yellow with a red-brown wash inside the throat of the lip and a red-brown spot on the lamina of the lip.

Sobralia gambitana is two times as tall as *S. tamboana* (ca 250 cm vs. 120 cm) while its floral segments are distinctly smaller than those of *S. tamboana* (60–66 mm vs. 78–92 mm). The two species differ also in the size of leaves and rachis of the inflorescence. The detailed comparison is presented in **Table 1**.

It looks like *S. tamboana*, but has relatively larger floral bracts and more slightly fractiflex inflorescence than *S. gambitana*.

3.1.1.11. *Sobralia tamboana* Dodson

Orquideología 21 (1): 44. 1998. Type: ECUADOR. Esmeraldas. Lita to San Lorenzo, Km 6, Alt. 650 m. 29

TABLE 1 Comparison of *Sobralia gambitana* Baranow, Szlach. and Kindlmann and *S. tamboana* Dodson.

Characters	*Sobralia gambitana*	*Sobralia tamboana*
Plant height	250 cm	120 cm
Leaves length	13–14 cm	26 cm
Leaves width	1.8–2.5 cm	8 cm
Inflorescence length	4 cm	12 cm
Ovary	10–11 mm	40 mm
Dorsal sepal size	66 mm × 22 mm	92 mm × 28 mm
Dorsal sepal shape	Lanceolate or elliptic-lanceolate	Narrowly oblong-elliptic
Dorsal sepal apex	Shortly acuminate	Acute
Lateral sepals size	60 mm × 20 mm	80 mm × 30 mm
Lateral sepals shape	Oblong elliptic	Obliquely oblong ovate
Lateral sepals apex	Shortly acuminate	Acute
Petals size	62 mm × 36 mm	78 mm × 30 mm
Petals shape	Obovate	Obliquely oblong-elliptic
Petals apex	Acute	Obtuse
Lip size	60 mm × 50 mm	80 mm × 40 mm
Lip shape	Rhombic-elliptic	Oblong-elliptic
Lip basal keels arrangement	United	Separated

December 1990. *C. H. Dodson and T. and P. M.* Dodson 19096 (Holotype: RPSC!; illustration of type).

Plants up to 120 cm tall, caespitose, rhizome short, stems cane-like, surrounded in the basal portion with clasping sheaths. Leaves up to 26 cm long and 8 cm wide, elliptic, chartaceous, acuminate at the apex, distichous, plicate, and heavily veined on the underside. Inflorescence ca 12 cm long, lightly flexuose with large, spathe-like bract at each node, the flowers produced singly in succession over prolonged periods with flowering concurrent throughout the population. Ovary ca 40 mm long. Flowers pale yellow with a red-brown wash inside the throat of the lip and a red-brown spot on the lamina of the lip. Sepals free to the base. Dorsal sepal 92 mm long, 28 mm wide, narrowly oblong-elliptic, acute. Lateral sepals to 80 mm long and 30 mm wide, obliquely oblong ovate, acute. Petals to 78 mm long and 30 mm wide, obliquely oblong-elliptic, obtuse, lightly reflexed at the apex, margins slightly crenate in the apical half. Lip up to 80 mm long and 40 mm wide, oblong-elliptic in general outline, upper half more or less deltoid, flared, retuse at the apex, concave, throat with a pair of shallow lamellae. Gynostemium 40 mm long, slender at the base, flattened on the underside, expanded on each side toward the apex to form falcate, horn-like stelidia (**Figure 11**).

Ecology: Epiphytic or terrestrial on road cuts and embankments. Flowering in March and December.

Distribution: Ecuador. Alt. 250–650 m.

Conservation status: EOO—CR, AOO—CR.

Representative specimens (**Supplementary Map** 10)—**Ecuador**. Lita to San Lorenzo, Km 6, Alt. 650 m, 29 December 1990. *C.H. Dodson and T., and P.M.* Dodson 19096 (RPSC!); Km 5, Lita to El Cristal, Alt. 250 m. 26 March 1993. *C.H. Dodson and G. Carnevali 19243* (RPSC!).

Similar to *Sobralia rosea* but distinguished by flexuose inflorescence, the pale yellow flowers with a diffuse red-brown spot and the lack of low, parallel lamellae on the lip lamina.

3.1.1.12. *Sobralia splendida* Schltr.

Repert. Spec. Nov. Regni Veg., Beih. **7**: 44. 1920. Type: Colombia. *Sine prec. loc. M. Madero* (B†).—Szlachetko et al. Materials to the Orchid Flora of Colombia **3**: 258. 2020.

Plants up to 300 cm tall, stem erect, leafy, growing in dense clumps, slightly compressed or subterete. Leaves 40–45 cm long, 9–10 cm wide, elliptic, acuminate, basally cuneate. Inflorescence 10–12 cm long. Rachis flexouose, glabrous. Flower color unknown. Floral bracts up to 90 mm long, lanceolate-cymbiform, acuminate. Ovary 40 mm long, cylindrical. Sepals basally connate together for one/fourth of their length. Dorsal sepal 85–90 mm long, 20 mm wide, oblong-ligulate to oblanceolate, acute. Lateral sepals 85–90 mm long, 20 mm wide, oblong-ligulate, acute, oblique. Petals 85–90 mm long, ligulate-oblanceolate, acute, slightly wider, and thinner in texture than the sepals, subfalcate. Lip 90 mm long, 40 mm wide, oblong ovate in outline above cuneate base, margins of apical half undulate, base with two parallel keels running up to its middle, the central vein in the central part of the lip ornamented with lamella, each of the protuberances with parallel rows of papillae on both sides. Gynostemium 57 mm long, slightly curved, apical stelidia oblong-falcate, not exceeding the anther (**Figure 12**).

Ecology: Terrestrial.

Distribution: Colombia. Alt. 500 m.

Representtive specimens—**Colombia**. Cauca. Alt. ca. 500 m. *M. Madero* (B†).

According to Schlechter (1920), this species is similar to *Sobralia ruckeri*, from which it differs by the lip structure, i.e., by the presence of the prominent, high papillae arranged in the rows running on both sides of each of the ridge of the lip. Our study supports his observations. The other species similar in lip form and gynostemium morphology to *S. splendida* is *S. hoppii*. In the former species, the lip base is ornamented with two lamellae running to its middle and the central vein in the central part is ornamented with lamella as well. In the latter, the lip has two basal keels, and the median vein is thickened, with two additional thickenings near the middle.

3.1.1.13. *Sobralia hoppii* Schltr.

Repert. Spec. Nov. Regni Veg., Beih. **27**: 13. 1924. Type (designated here): Colombia. Caqueta. Ostkordillere, Putumayo-Gebiet, Alt. 3000 m. September 1922, *W. Hopp 164* (B†); Von Buenaventura bis Juntas. Alt. to 300 m. 21 July

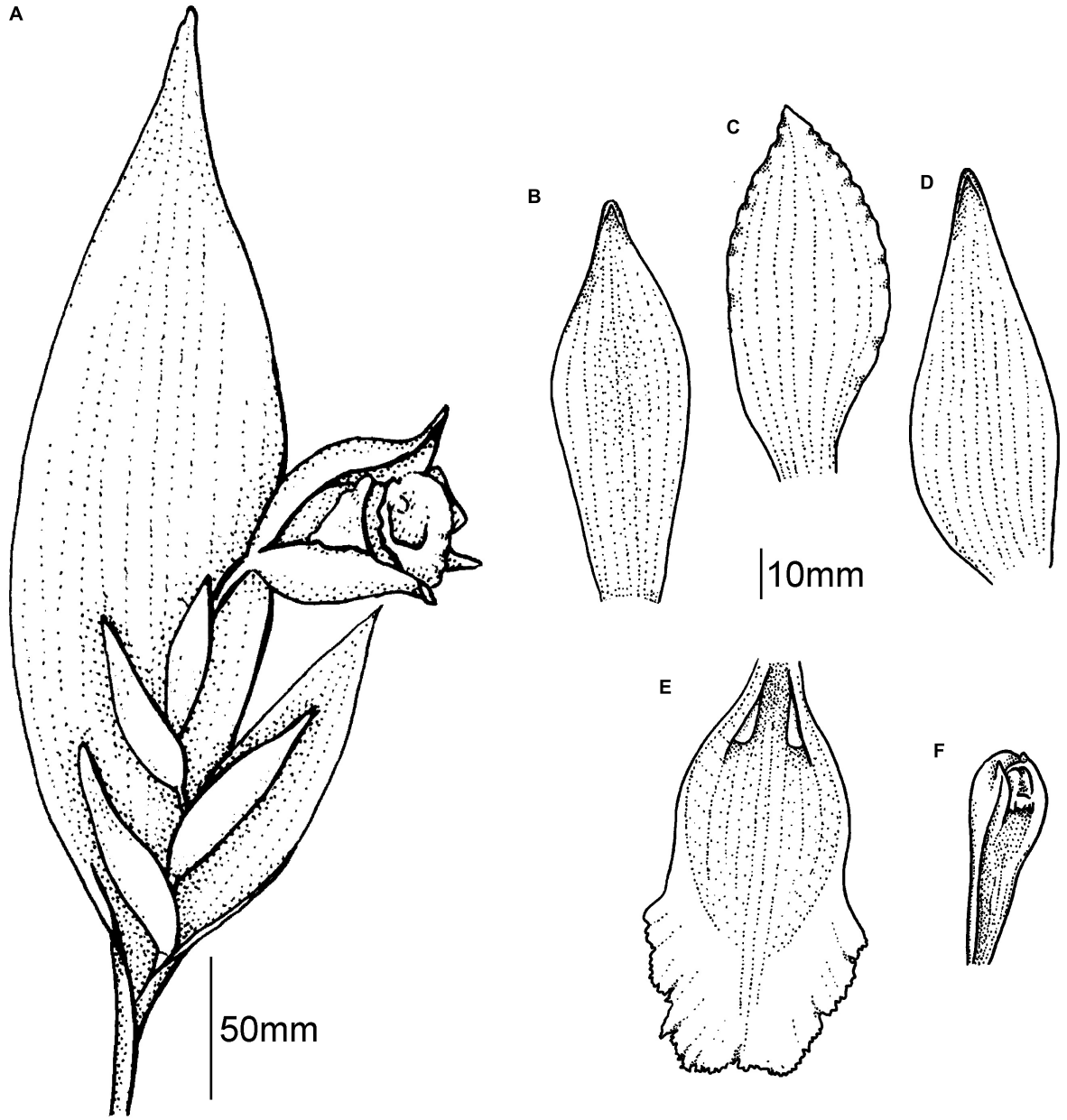

FIGURE 11
Sobralia tamboana Dodson **(A)** apical part of stem with inflorescence, **(B)** dorsal sepal, **(C)** petal, **(D)** lateral sepal, **(E)** lip, and **(F)** gynostemium, front view [drawn by P. Baranow from Dodson (1998)].

1881. *W. Hopp 753* (Neotype: W!, UGDA-DLSz!–drawing).—Szlachetko et al. Materials to the Orchid Flora of Colombia **3**: 259. 2020.

Plants probably 150 cm tall, erect, robust, glabrous. Leaves 23–30 cm long, 7.5–11 cm wide, elliptic, acuminate, many-veined, coriaceous, stiff. Raceme up to 35 cm long, 5–12-flowered, rachis flexuose, glabrous, or sparsely furfuraceous. Flowers rather large, pure white or yellowish-white. Floral bracts up to 130 mm long, ovate, long-acuminate. Ovary 32 mm long, glabrous. Dorsal sepal 60–83 mm long, 10–14 mm wide, oblong-ligulate, acuminate. Lateral sepals 60–83 mm long, 10–14 mm wide, obliquely oblong-ligulate, acuminate. Petals 52–83 mm long, 22 mm wide, obliquely oblong, obtuse to subobtuse, with more or less undulate margins. Lip 52–85 mm long in total, 30–40 mm wide when expanded, unguiculate, ovate to oblong ovate in the general outline above, more or less pandurate toward apical quarter, emarginate, undulate in front, with 2 basal keels, median vein thickened, with two additional thickenings near the middle. Gynostemium 37–67 mm long, stelidia

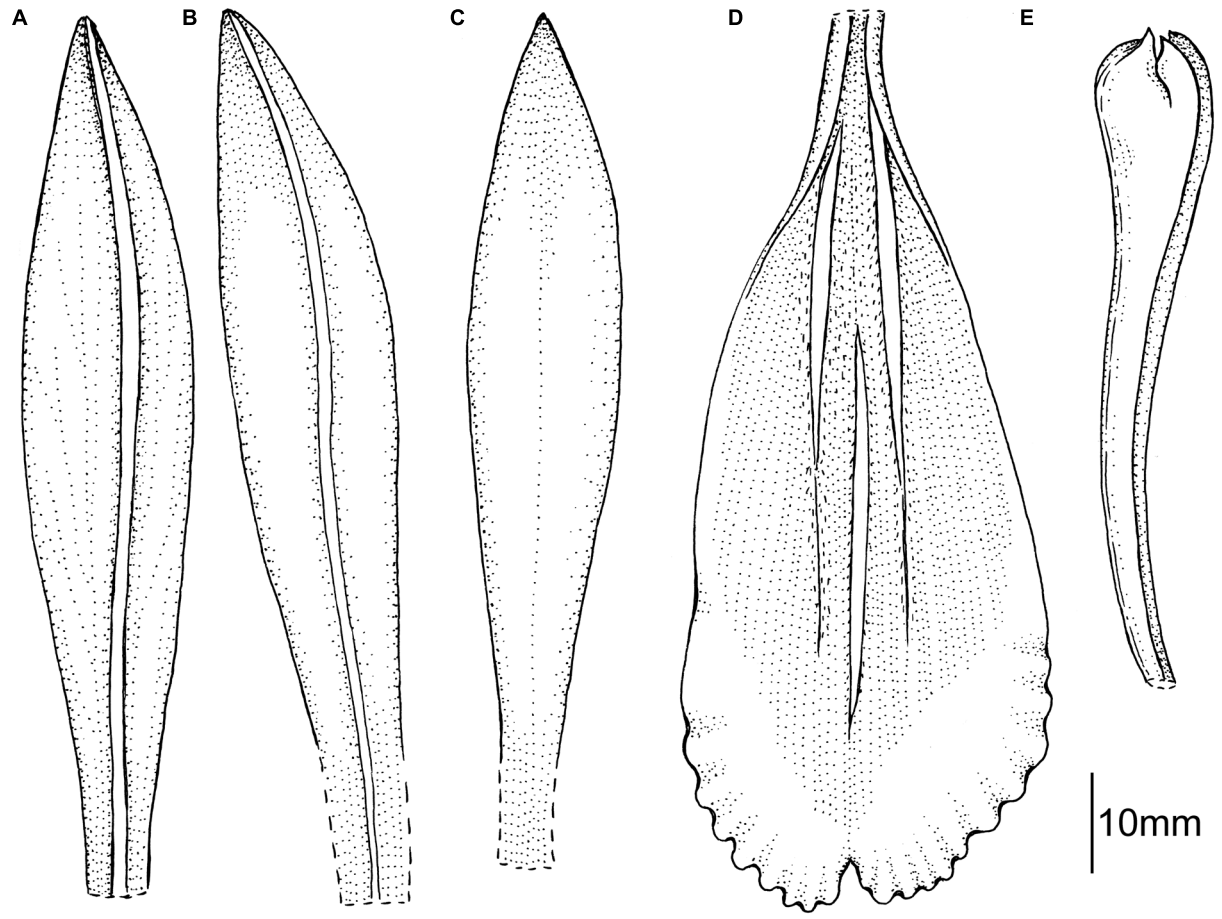

FIGURE 12
Sobralia splendida Schltr. **(A)** dorsal sepal, **(B)** lateral sepal, **(C)** petal, **(D)** lip, and **(E)** gynostemium, side view [redrawn by A. Król from Schlechter (1929)].

relatively obscure, obliquely triangular, shorter than anther (**Figure 13**).

Ecology: Terrestrial. Flowering in May, July, and in September.

Distribution: Colombia. Alt. 300_metricconverterProductID3000 m–3000 m.

Conservation status: EOO—CR, AOO—CR.

Representative specimens (**Supplementary Map 11**)—**Colombia**. Caquetá. Putumayo-Gebiet, Ostkordillere. Alt. 3000 m. September 1922. *W. Hopp 164* (Schlechter, 1924). Chocó. Mpio. Carmen del Atrato. Carretera Quibdó-Carmen del Atrato. 5°43.6′-43.5′N, 76°36.2′-18.4′. Alt. 80–510 m. 11 May 2007. *R. Arevalo, J. Betancur, S. Hoyos, and E. Renteria 740* (COL!). Valle del cauca. Von Buenaventura bis Juntas. Alt. to 300 m. 21 July 1881. *W. Hopp 753* (W!, UGDA-DLSz!–drawing).

According to Schlechter (1924), this species resembles *Sobralia rosea* and can be easily misidentified with it, but has smaller, pure white or yellowish-white flowers. In the form of the lip and its callosities, the species is easily distinguishable from all other taxa of this group.

As the original collection is not available—we assume it could have been destroyed during World War II—we decided to designate the neotype for the species. We have chosen the only existing collection of the species gathered by Hopp (no. 753), who was also the collector of the original type material. Besides, the selected collection is well documented by the drawings left in Vienna and UGDA herbaria.

3.1.1.14. *Sobralia pulcherrima* Garay

In Harling & Sparre, Fl. Ecuador **9**: 128. 1978. Type: Ecuador. Pichincha: road Nanegal–Nanegalito, Alt. 1200–1550 m. *G. Harling and L. Andersson 11571* (Holotype: GB; Isotype: AMES 00104326; K–drawing!).—Szlachetko et al. Materials to the Orchid Flora of Colombia **3**: 260. 2020.

= *Sobralia lindenii* Grignan, Lindenia **13**: t. 5855. 1895, *not hort*. Type: *no data*.

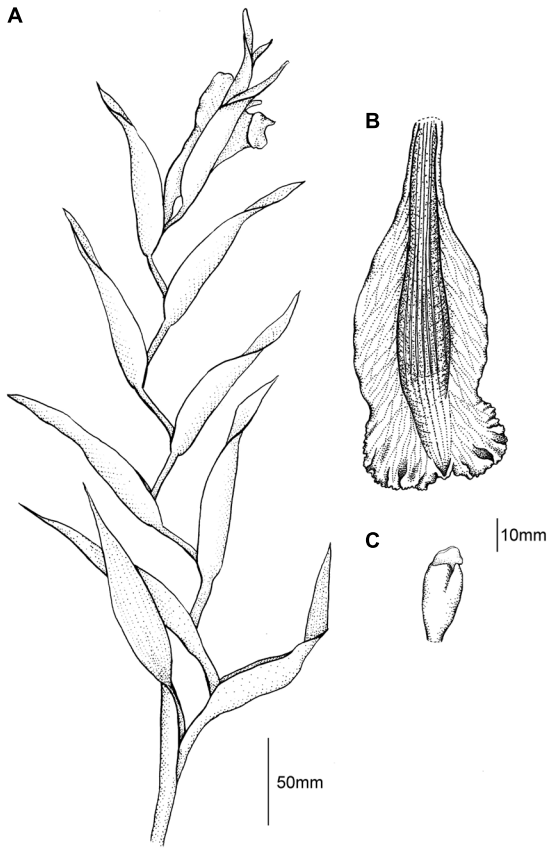

FIGURE 13
Sobralia hoppii Schltr. **(A)** apical part of stem with the inflorescence, **(B)** lip, and **(C)** apical part of the gynostemium, side view (drawn by P. Baranow from the neotype).

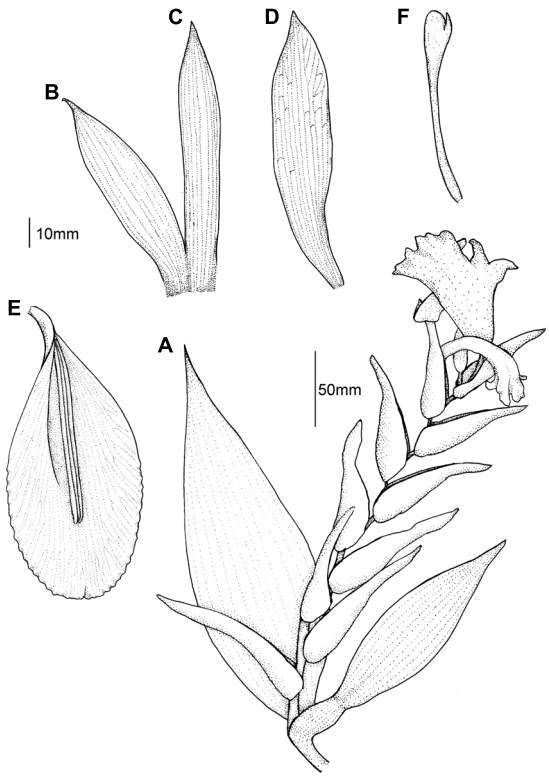

FIGURE 14
Sobralia pulcherrima Garay **(A)** apical part of stem with inflorescence, **(B)** lateral sepal, **(C)** dorsal sepal, **(D)** petal, **(E)** lip, and **(F)** gynostemium, side view (drawn by P. Baranow from *Asplund 16728*).

Plants up to 400 cm tall, caespitose. Stem erect, rather robust, lower half leafless, completely enclosed by remnants of leaf sheaths, leafy above. Leaves up to 33 cm long and 9 cm wide, ovate-lanceolate or elliptic-lanceolate, long-acuminate, gradually tapering to a more or less rounded base, sessile and articulated with glabrous sheaths, plicate. Inflorescence up to 30 cm long, sessile, elongating with age, flexuous, loosely few-flowered. Flowers 1 or 2 at a time produced in succession, large, showy, white with dark purple veins on the lip disk. Floral bracts up to 100 mm long, ovate-lanceolate, cymbiform, erectly spreading with arcuate tips. Pedicellate ovary up to 30 mm long. Dorsal sepal up to 105 mm long and 30 mm wide, oblanceolate-oblong, subfleshy, acute or abruptly acuminate, somewhat tapering toward the base, more or less undulate, connate with lateral sepals for up to 15 mm. Lateral sepals up to 105 mm long and 30 mm wide, lanceolate to oblanceolate-oblong, subfleshy, acute or abruptly acuminate, somewhat tapering toward the base, margins undulate. Petals up to 105 mm long and 30 mm wide, oblanceolate-obovate, acute, subfalcate, with more or less undulate margins. Lip up to 115 mm long and 65 mm wide, ovate-elliptic in general outline, with a tubular base, then flabellate spreading in front, very undulate-crispate, bilobed in front with erose denticulate margin, disk with 3 lamellae running from the base to the middle, the median lamella erect, high-carinate, the lateral ones appressed to the disk, on both sides of lamellae veins thickened and barbate. Gynostemium up to 65 mm long, clavate, arcuate, bifalcate, stelidia as long as anther (Figure 14).

Ecology: Terrestrial in lowland and premontane forest edges. Flowering throughout the year.

Distribution: Ecuador, Colombia. Alt. up to 2000 m.

Conservation status: EOO—LC, AOO—EN.

Representative specimens (Supplementary Map 12)—**Ecuador**. Carchi. Approx. 3 km above Maldonado. Alt. 1550 m. *B. Boyle and J. Bradford 1854* (MO!); Maldonado to Chical, km 3. Alt. 1410 m. 30 April 1993. *C.H. Dodson 19084* (RPSC!). Esmeraldas. Lito to San Lorenzo, km 4. Alt. 230 m. 26 March 1994. *C.H. Dodson and G. Carnevali 19235* (RPSC!). Pastaza. Puyo-Napo road. 11–18 October 1975. *P.M. Synge 9* (K!). Pichincha. near the bridge over the Río Pilaton between Chiriboga and Santo Domingo de

los Colorados. Alt. 1100 m. 1 July 1955, *E. Asplund 16728* (AMES!); About 65 miles SW of Quito. 12 November 1969. *P. Clark s.n.* (F!); Road Nanegal to Nanegalito. Alt. 1200–1550 m. *G. Harling and L. Andersson 11571* (GB; AMES!, K–drawing!); Santo Domingo-Quito Road, 8 km northeast of Santo Domingo. Alt. 76 m. 29 July 1980. *R.P. Sauleda, M. Ragan, H. Luther, R. Wunderlin, B. Hansen, L. Davenport,and J. Wiersema 3799* (AMES!, MO!, U!); Route Tandayapa-Nenegalito, Fundacion Maquipucuna, 00°00′S, 78°40′W. Alt. 1400 m. 24 January 1996. *F. Billet and B. Jadin 6700* (MO!). Zamora-Chinchipe. Cordillera del Cóndor, vertiente occidental. Cuenca del Río Tundayme. Carretera hacia el destacamento militar Condor Mirador. Formacion rocosa arenisca, suelo arenoso. 3°37′48″S, 78°26′50″W. Alt. 1690–2000 m. 21 March 2006. *W. Quizhpe and F. Luisier 2034* (MO!).

Colombia. Chocó. Road between Medellín and Quibdó at km 134.5. 5°46′N, 76°20′W. Alt. 1070 m. 13 April 1983. *T.B. Croat 55918* (MO!); Carretera Tutunendo-El Carmen. Entre km 135 y 120. Alto Río Atrato. Alt. 800–1200 m. 29 April 1979. *E. Forero, R. Jaramill M., H.Y. Bernal, H. Leon, and M.M. Pulido 6091* (COL!, P!); Hoya del Río San Juan. Arriba de Palestina, entre Quebrada La Sierpe (Palestina) y Quebrada El Quicharo. 4°10′N, 77°10′W. 27 Mar. 1979. *E. Forero, R. Jaramillo M., L.E. Forero P., and Hernandez N. 4103* (COL!, MO!); Río Yuto between Lloró and La Vuelta. Alt. 100 m. 18 January 1979. *A. Gentry and E. Renteria A. 17426* (COL!); Ca 15 km W of Siete. 6 January 1979. *A. Gentry and E. Renteria A. 23718* (COL!, MO!); Río Yuto between Lloró and La Vuelta. Alt. 100 m. 18 January 1979. *A. Gentry and E. Renteria A. 24348* (COL!, P!); Hwy. Bolivar-Quibdó, near km 135, 5°50′N, 76°20′W. Alt. 975 m. 28 October 1983. *A. Juncosa 1122* (MO!, NY!). Valle del Cauca. Mpio Dagua. Corregimientoo El Danubio, Alto Anchicaya. Alt. 200 m. 19 June 1984. *W. Devia A. 568* (MO!); Río Anchicaya near CVC hydroelectric plant, 3°40′N, 76°50′W. Alt. 400–500 m. *A. Gentry 35656* (COL!, MO!); Carretera from Buenaventura to Cali, km 20. 4 June 1982. *H. Murphy 573* (COL!, MO!); Along the road El Queremal-La Elsa. On steep slopes. 15 February 2011. *D. Szlachetko, A. Niessen and M. Moreno s.n.* (UGDA-DLSz–spirit!); Between Buenaventura and Cali on old highway, 5 km S of Río Sabaletas along steep soggy bank along road, 3°44′N 76°57′W. Alt. 145 m. 10 February 1990. *T.B. Croat and J. Watt 70413* (CUVC!, MO).

This species resembles *Sobralia rosea* but can be distinguished by the flower color—*S. pulcherrima* always has a white lip with broad white margins and the disk is prominently purple-veined. *S. rosea* is always with a narrow, white margin while the whole disk is crimson-purple with white radiating veins. *S. pulcherrima* is limited in distribution to the western foothills of the Andes, while *S. rosea* can be found on the eastern foothills of the Andes. Both species while pressed and dried out can be separated by the lip details. The lip disk of *S. pulcherrima* has three lamellae running from the base to the middle, and the median one is high-carinate. On the contrary, the lip disk of *S. rosea* from the base to the center is transversed by 5–7 low, parallel ridges, with fine, radiating, white veins in the center.

3.1.1.15. *Sobralia rosea* Poepp. & Endl.

Nov. Gen. Sp. Pl. **1**: 54, t. 93. 1836. Type (designated by Szlachetko et al.:261. 2020): Peru. *Sine loc. E.F. Poeppig 1076* (Lectotype: W! 47809, Isolectotype: W! 47808).–Schweinfurth. Orchids of Peru 74. 1958.–Garay in Harling & Sparre. Fl. Ecuador. Orchid. 9: 133. 1978.—Szlachetko et al. Materials to the Orchid Flora of Colombia **3**: 261. 2020.

=*Sobralia lindenii* Hort., Gard. Chron. **18**: 424. 1895. Type: Introduced from tropical America, flowered by *T. Lawrence* in 1894 and *C. J. Lucas* in 1895 (Holotype: K!).

Plants up to 150 cm tall. Stem erect, robust, cane-like, leafless below, many-leaved above. Leaves up to 35 cm long and 8 cm wide, lanceolate, long-acuminate, sessile on glabrous sheaths. Inflorescence up to 15 cm long, sessile, few-flowered, flexuosus. Flowers 1 or 2, produced in succession, rather thin in texture, pale rose color, the main disk of lip dark purple–magenta with narrow white margin and transversed by white veins. Floral bracts up to 90 mm, cymbiform, ovate, acute to acuminate. Pedicellate ovary up to 25 mm long, cylindric, glabrous. Dorsal sepal up to 100 mm long and 20 mm wide, narrowly oblanceolate, acute, dorsally fleshy, subulate, basally connate with lateral sepals for up to 10 mm. Lateral sepals up to 100 mm long and 20 mm wide, narrowly oblanceolate, acute, dorsally fleshy, subulate. Petals up to 100 mm long and 25 mm wide, oblanceolate-elliptic, obtuse to acute, with somewhat undulate margin. Lip up to 110 mm long and 55 mm wide, oblong ovate to ovate-elliptic in outline, tubular in a natural position, then expanding in a suborbicular, bilobed, frontal blade, when expanded, from a cuneate base obovate, undulate, crispate in front, retuse to deeply bilobed at apex, disk transversed in center by 5–7 low, parallel ridges which are confluent at base, where on both sides subpubescent or papillose. Gynostemium up to 55 mm long, clavate, arcuate, stelidia shorter than anther (**Figure 15**).

Ecology: Terrestrial in forest edges and steepy river sides. Flowering throughout the year.

Distribution: Ecuador, Colombia, Peru, Brazil. Alt. from sea level up to 3300 m.

Conservation status: EOO—LC, AOO—EN.Representative specimens (**Supplementary Map 13**)—**Ecuador**. Azuay. Cola de San Pablo, Norriente de Paute en el Río Paute. Alt. 1300 m. 9

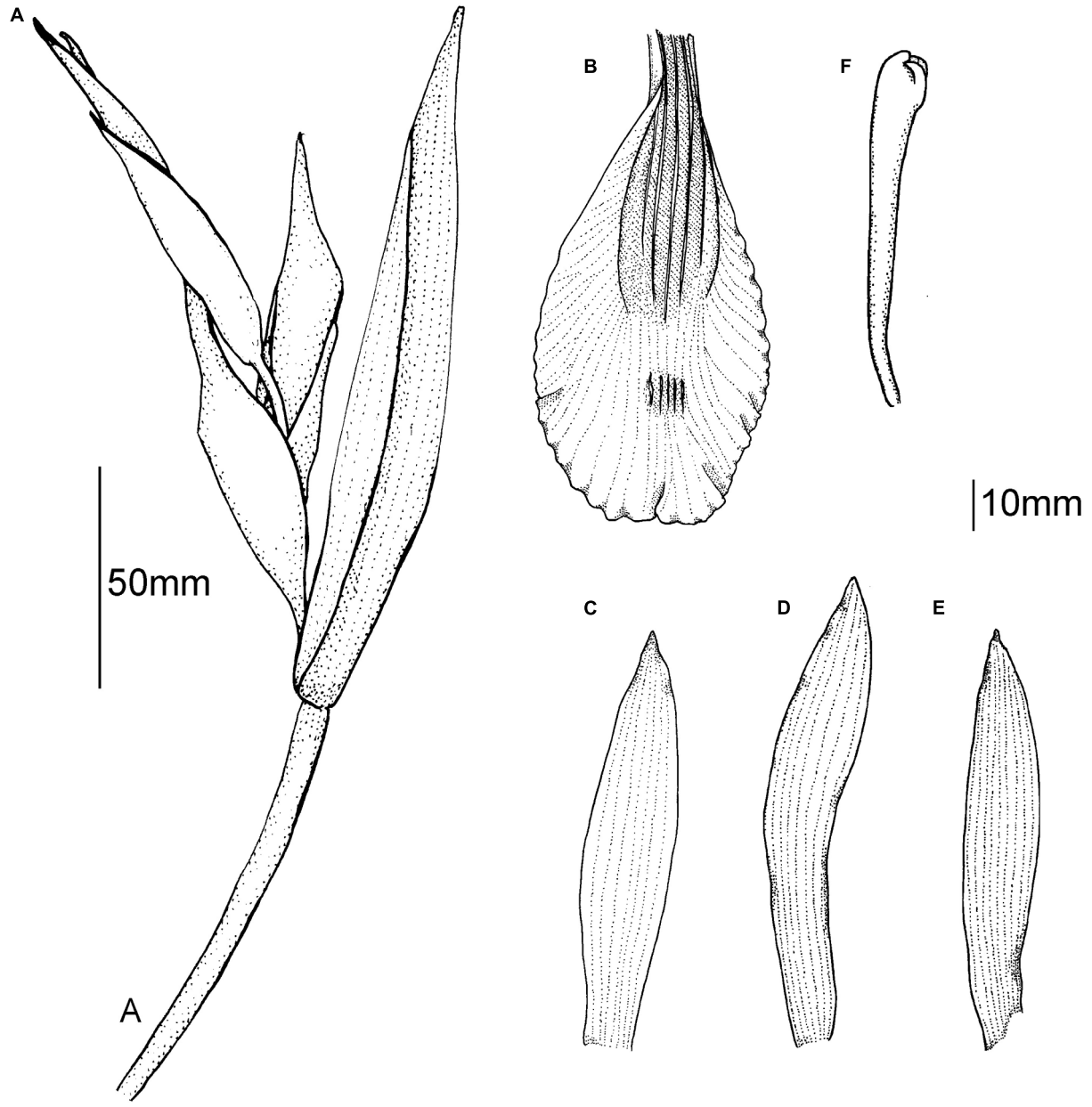

FIGURE 15
Sobralia rosea Poepp. and Endl. **(A)** apical part of stem with inflorescence, **(B)** lip, **(C)** dorsal sepal, **(D)** petal, **(E)** lateral sepal, and **(F)** gynostemium, side view (drawn by P. Baranow from lectotype).

March 1985, *C. and P. Dodson, C. and J. Luer, and A. Hirtz 15779* (RPSC!). Esmeraldas. Along Río Lita in the vicinity of the village of Lita. Alt. 600–650 m. 8 September 1976, *T.B. Croat 38937* (MO!); Km 11 Lita to San Lorenzo. Alt. 760 m. 12 May 1990, *C.H. Dodson, A. Gentry, B. Boyle, and D. Rubio 18241* (RPSC!); Along road under construction from Lita to Alto Tambo (21 km). Alt. 750–820 m. 19 May 1987, *C.H. Dodson, H. van der Werff, and W. Palacios 17130* (RPSC!). Los Rios. Quevedo-Latacunga road, km 46 from Quevedo, 79°11′W. 0°55′S. Alt. 600 m. 4 April 1973, *L. Holm-Nielsen, S. Jeppesen, B. Lojtnant, and B. Ollgaard 2896* (AMES!, K!, MO!). Carchi. Road Tulcán to Maldonado *via* Paramo El Angel, km 74. Alt. 1750 m. 1 August 1985, *C.H. Dodson and A. Embree 16192* (RPSC!); Between Chical and Peñas Blancas trailside *and* forest edge, valley of San Juan on Colombia border. Alt. 1100–1250 m. 25 September 1979, *A. Gentry and G. Shupp 26476* (MO!).

Cotopaxi. Tenefuerte, km 52 Quevedo-Latacunga. Alt. 800–900 m. 9 April 1984, *C.H. Dodson and W. and M. Thurston 14216* (RPSC!); Tenefuerste. Río Pilalo, km 52–53, Quevado, Latacunga. Alt. 750–1300 m. 21 February 1982, *C.H. Dodson and A.H. Gentry 12726* (RPSC!). Morona-Santiago. Indanza-Limón (General Plaza). Alt. 1300–1600 m. 23 March 1974, *G. Harling and L. Andersson 12753* (AMES!). Napo. Reserva Biologica Jatun Sacha. Río Napo, 8 km al. E de Misahuallí. 1°04′S, 77°36′W. Alt. 450 m. 24 April–5 May 1987, *C.E. Ceron M. 1302* (MO!); Laguna Anangu, N side, 00°31′S, 76°24′W. Alt. 250 m. 25 January 1985, *B. Ollgaard 57158* (MO!). Napo-Pastaza. Valley of Río Pastaza and adjacent uplands. Alt. 1060–1500 m. 17 April 1945, *W.H. Camp E-2382* (AMES!); Mera. 1 March 1940, *H. Lugo M. 7* (B!, MO!). Pastaza. Along the highway between Shell and Mera. Alt. 1000 m. 18 March 1988, *B. Boom and D. Beardsley 8442* (US!); Pastaza Canton, Estacion experimental Pastaza, *via* Puyo–Macas, Trama km 31.5–33 Puyo Macas, borde del carretero. 1°30′S, 77°56′W. Alt. 1040 m. 16 February 2002, *J. Caranqui, M. Melampy, and J. Lara 399* (MO!); Cantón Arajuno, bosque protector Pablo Lopez del Oglan Alto y Estacion Cientifica de la Universidad Central de Ecuador, 1°19.25′S, 77°41.19′W. Alt. 600 m. 5 March 2006, *C.E. Ceron, C.I. Reyes, and L. Marcelo Vargas 56657* (MO!); Along the road between Puyo and Baños, 2.7 km W of Mera, 4.6 km W of Shell, 1°27′S, 78°50′W. Alt. 1110 m. 5 May 1984, *T.B. Croat 59084* (MO!); Puyo-Puerto Napo road. 25 December 1972, *R.H. Williamse 16* (U!); Hacienda San Antonio Baron von Humboldt, 2.5 km Norte de Mera en carretera a Baños-Puyo. Alt. 1050–1300 m. 23 March 1985, *C.H. Dodson and L.M. Bermeo 15604* (AMES!, K!, MO!); Mara, road cut near Mangayacu. Alt. 1100 m. 28 January 1956, *E. Asplund 19085* (AMES!, B!, K!); Hacienda San Antonio Baron von Humboldt, 2 km al. Norte de Mera, 1°27′S, 78°06′W. Alt. 1100 m. 20 February 1985, *W. Palacios, M. Baker and J. Zaruma 62* (RPSC!). Pichincha. El Chaupi, along the road to Iliniza. Alt. 3300 m. 19 April 1967, *B. Sparre 15645* (US!); Los Rios. Km 90, Camino Viejo *via* Chiriboga, Quito-Santo Domingo. Alt. 1100 m. 7 April 1984, *C.H. Dodson and W. and M. Thurston 14173* (RPSC!); along the river just outside the town of Mindo on the new road to Liloa. Alt. 1300 m. *C.H. Dodson, E. Hagsater, and A. Hirtz 16669* (RPSC!); Km 40–51 on road Santo Domingo de los Colorados-Quito, forested slopes along Río Pilaton, 0°55′S, 78°55′W. Alt. 1100–1400 m. 14 June 1973, *L. Holm-Nielsen, S. Jeppensen, B. Lojtnant, and B. Ollgaard 7154* (AMES!). Sucumbíos. Río San Miguel o Sucumbios, Santa Rosa y los alredadores. Alt. 380 m. 7–8 April 1942, *R.E. Schultes 3559* (COL!); Tungurahua. Río Verde Grande. Alt. 1500 m. 30 March 1956, *E. Asplund 20049* (AMES!); Baños-Puyo, km 35. 1°24′S, 78°12′W. Alt. 1170 m. 11 February 1978, *P. Bamps 6232* (MO!); Between Baños and Río Verde. Alt. 1680 m. 29 April 1951, *P. R. Bell 812* (BM!); Valley of Pastaza River, between Baños and Cashurco, 8 h east of Baños. Alt. 1300–1800 m. 25 September 1923, *A.S. Hitchcock 21754* (AMES!; US!); Río Estancias, near Río Negro, southern side of Río Pastaza, 3 March 1969, *H. Lugo S. 621* (AMES!, MO!); Along Pastaza River below Machay. Alt. 1350 m. 18 March 1939, *C. W. Penland and R.H. Summers 113* (AMES!); Río Verde. 21 April 1971, *H. Lugo S. 1770* (AMES!, MO!); Along road from Baños to Puyo from Río Blanco to Puyo. Alt. 700–1800 m. 23 February 1963, *L.B. Thien 2302* (F!). Zamora-Chinchipe. Road Loja-Zamora, km 54, 78°59′W. 4°02′S. Alt. 1300 m. 18 April 1973, *L. Holm-Nielsen, S. Jeppesen, B. Lojtnant, and B. Ollgaard 3774* (AMES!, K!, MO!); Río Negro, Rd. Baños-Puyo. Alt. 1500 m. 15 October 1984, *C.H. and P.M. Dodson, and A. Hirtz 15370* (RPSC!); Road Loja to Zamora, km 48. Alt. 1400 m. 17 May 1867, *B. Sparre 16344* (US!). Zamora-Chinchipe. Road Loja-Zamora, El Retorno-Zumbi. Alt. 1000 m. May 1985, *D. Dalessandro 460* (RPSC!). **Colombia**. Antioquia. Hillsides near Puente Linda, 5 km above Río Samana. Alt. 1000 m. 26 July 1960, *F.A. Barkley and G. Gutierrez V. 35345* (AMES!); Río Grande. April 1947, *Bro Daniel 4000* (US!). Cauca. El Tambo, Parque Nacional Natural Munchique, vereda La Romelia, la Gallera. Alt. 2835 m. 26 July 1993, *C. Barbosa et al. 8588* (COL!, MA!). Chocó. Río San Juan, cercenias de Palestina. Alt. 5–50 m. 12–14 March 1944, *J. Cuatrecasas 16942* (AMES!; F!); Km 55 de la carretera Ansermanueve-San José del Palmar. Alt. 1700–1950 m. 19 March 1980, *G.C. Lozano and J. Diaz 3229* (COL!, F!). Nariño. Mpio Tumaco. La Guayacana. 27 June 1951, *R. Romero Castañeda 2909* (COL!, MO!). Mpio. Barbacoas. Chucunes *via* La Planada a 1 km antes de llegar a la reserve. Alt. 1800 m. 10 March 1995, *G. Lozano, J.L. Fernadez Alosno, and E. Morales 6878* (COL!); Mpio. Barbacoas. Correg. Junin. *Via* Junin-Barbacoas. Alt. 960–1100 m. 14 March 1995, *G. Lozano, J.L. Fernadez Alosno, and E. Morales 6977* (COL!); Barbacoas. Corregimiento Santander (Buenavista) a Barbacoas (Vertiente del Río Telembi). Alt. 840 m. 3–5 August 1948, *H. Garcia Barriga 13188* (COL!); Km 68 del Ferrocarril Tumaco-El Diviso. 28 July 1952, *R. Romero Castañeda 3334* (COL!); Frontera Colombo-Ecuatoriana. Selva higrofila del Río San Miguel. Margenes del Río entre los afluentes Churruyaco y Bermejal. Alt. 350–400 m. 12 December 1940, *J. Cuatrecasas 11015* (COL!). Putumayo. Valle de Sibundoy. Alt. 2500–3000 m. 1963, *C. Krauss 51* (COL!); Río Pepino, carretera a 10 km de Mocoa. Bosque alto. Alt. 850 m. 6 January 1957, *M. Ospina H. 117a* (COL!); Margenes del Río Guamues entre San Antonio y la desembocadura, 20 December 1940, *J. Cuatrecasas 11220* (COL!); Vertiente oriental de la cordillera, entre Sachamates y San Francisco de Sibundoy, Planada de Minchoy. Alt. 2100 m. 30 December 1940, *J. Cuatrecasas 11439* (F!, US!); Entre San Francisco y El Pepino. Alt. 1900–2400 m. 2 August 1961, *A. Fernandez-Perez 5853* (COL!); Mpio Villa Garzón. Carretera a Puerto Asis. 1°10′N, 76°34′W. Alt. 1350 m. 3–4 May 1994, *J.L. Fernandez A., A. Camero, and E. Mesa 11467* (COL!, MO!); Río Pepino, carretera a 10 kms de Mocoa. Alt. 850 m. 6 January 1957, *M. Ospina H. and J.M. Idrobo 117* (AMES!); Mpio. Mistrató. Hacia San Antonio del Chami. Quebrada Sutu y Empalados. Alt. 1700–1800 m. 26 April 1992, *G.C. Lozano*

and Estudiantes Introduccion Systematica 6382 (COL!); Cerro de Portachuelo, entre Mocoa y Sacchamates. Alt. 1600–2000 m. 9 December 1942, *R.E. Schultes and C.E. Smith 3049* (COL!, K!, NY!, US!). Valle del Cauca. Costa del Pacifico, Río Cajambre, Barco. Alt. 5–80 m. 21–30 April 1944, *J. Cuatrecasas 17250* (AMES!, COL!, F!); Chichito, Western Cordillera. Alt. 1600 m. November 1937, *E. Dryander 1994* (US!); Wooded cliffs of Río Dagua. Alt. 80–100 m. 6–8 May 1922, *E.P. Killip 5057* (AMES!, NY!); Boca del Lobo, Buenaventura Bay. 9 June 1944, *E.P. Killip and J. Cuatrecasas 38985* (F!, US!); Km 80 Cali-Buenaventura. Alt. 350 m. 1 July 1965, *C.H. Dodson and H. Hills 3215* (F!); Cerca a la Elsa. Alt. 1250 m. 5 August 1966, *S. Espinal T. 1903* (AMES!, CUVC!); Queremal, Crece el Saludos. 20 January 1980, *I. Guarin O. 63* (COL!); New road Cali-Buenaventura, La Pesuòa, 14 February 2011, D. *Szlachetko, C. Uribe, and M. Moreno 9036* (UGDA-DLSz–spirit!). **Venezuela**. Táchira. Between la Providencia and San Vicente de la Revancha, southwest of Santa Ana. Alt. 1650 m. 8 January 1968, *J.A. Steyermark and G.C.K. and E. Dunsterville 100533* (AMES!). **Peru**. Amazonas. Bagua Prov. Yamayakat bosque de Rivera. 4°55′S, 78°19′W. Alt. 320 m. 31 January 1996, *N. Jaramillo, M. Jaramillo, and D. Chamit 1024* (MO!); Bagua Distr. Aramango, Soldado Oliva, 5°18′S, 78°20′W. Alt. 600 m. 6 February 1999, *R. Vasquez, C. Vargas C., J. Yactayo, and E. Palomino 26046* (MO!); Central Cordilleras of the Andes. Alt. 2700–3300 m. 30 March 1938, *L. Williams 7603* (AMES!, F!). Cusco. Marcapata. Alt. 2000 m. 24 July 1957, *C. Vargas C. 1168* (CUZ, F!); Cardena. Alt. 1020 m. 29–30 July 1946, *C. Vargas C. 6194* (F!); Maniri. Alt. 1200–1900 m. 8 December 1962, *C. Vargas C. 14064* (CUZ, F!). Huánuco. Cuchero, *Sine loc*. 1829, *E.F. Poeppig s.n.* (W! 47810); Bajando de Carpish a Tingo María. Alt. 2700–2900 m. 5 March 1947, *R. Ferreyra 1817* (AMES!). Huánuco. Pampayacu. Hacienda at mouth of Chinchad Rio, Alt 3500′, 19–25 July 1923, *J.F. Macbride 5017* (F!). Junin. Colonia Perené. Alt. 680 m. 30 March 1938, *E.P. Killip and A.C. Smith 24948* (AMES!; F!, US!); Satipo Prov. Gran Pajonal, Chequitavo, 10°45′S, 74°23′W. Alt. 1200 m. 27 March 1984, *D.N. Smith 6544* (MO!); Prov. Huánuco, Highway La Oroya–Tingo María, km 66 east of Huánuco. Alt. 1620 m. 8 March 1977, *J.D. Boeke 1167* (MO!). Oxapampa. Cueva Grande, Estacion near Pozuzo. Alt. 3500′, 23 June 1923, *J.F. Macbride 4804* (F!). San Martín. Boqueron Pass, 92 km from Tingo María on highway to Pucallpa. Alt. 400 m. 16 December 1949–5 January 1950, *H.A. Allard 2755* (US!); Tingo María. Alt. 625–1100 m. 30 October 1949–19 February 1950, *H.A. Allard 22567* (US!); Across Río Tocache from Tocache Nuevo, road to Juanjuí. Alt. 500 m. 16 July 1982, *A. Gentry, D. Smith and R. Tredwell 37640* (MO!); near Mayobamba. Alt. 1200–1600 m. March 1934, *G. Klug 3602* (AMES!, F!, K!, MO!); Prov. Rioja, Rioja, Salida a Mashoyacu-Shucaqai. Bosque protection Amto Mayo, Toma de Agua, Quebrada Cuchachi, Alt. 1000 m. 15 July 1995, *I. Sanchez Vega 8052* (F!); Prov. Mariscal Caceras Dtto. Tocacho Nuevo (Muyuna de Huayrurillo) (margen derecha del Río Huallaga), 10 March 1971. *J. Schunke V. 4753* (B!, F!, US!); Mariscal Cáceres, Tocache Nuevo. Camino al. Caserio de Santa Rosa de Mishollo, 4 km de Puerto Pizana, 20 May 1971, *J. Schunke V. 4916* (F!); San Roque. Alt. 1350–1500 m. January–February 1930, *L. Williams 7795* (F!).

When *Sobralia lindeni* was described in 1895 from cultivated material upon which the description was based represented undoubtedly *S. rosea*. As a matter of fact, because of the great similarity in the general appearance of this species and *S. pulcherrima* the two have been combined in Lindenia in 1897 under *S. lindeni* as representing two distinct forms, those with white flowers and those with pale lilac or rose-colored flowers. The white-flowered form is the true *S. pulcherrima* (Garay, 1978).

3.2. *Incertae sedis*

3.2.1. *Sobralia augusta* Hoehne

Arq. Bot. Estado São Paulo 1: 128. 1944. Type: Brazil. Mato Grosso, Rio Juruena, Salto augusta, February 1912, *F.C. Hoehne 5349* (SP).

The only material devoted to *S. augusta* that we could study is the drawing publisher in Flora Brasilica (Vol. XII, Table 51). Based on the illustration, we can suspect, that taxon is a synonym of *S. liliastrum*. However, until the type material will be available for analysis, we decide not to change its taxonomic status.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

PB: herbarium material revision, data gathering, analysis of distribution, manuscript writing, figures, and maps. DS: herbarium material revision, data analysis, and manuscript writing. PK: analysis of the results and manuscript writing and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.1058334/full#supplementary-material

SUPPLEMENTARY MAP 1
Distribution map of *Sobralia paradisiaca* Rchb.f.

SUPPLEMENTARY MAP 2
Distribution map of *Sobralia chrysantha* Lindl.

SUPPLEMENTARY MAP 3
Distribution map of *Sobralia liliastrum* Lindl.

SUPPLEMENTARY MAP 4
Distribution map of *Sobralia elisabethae* R. H. Schomb.

SUPPLEMENTARY MAP 5
Distribution map of *Sobralia granitica* G.A. Romero & Carnevali.

SUPPLEMENTARY MAP 6
Distribution map of *Sobralia gambitana* Baranow, Szlach. & Kindlmann.

SUPPLEMENTARY MAP 7
Distribution map of *Sobralia luerorum* Dodson.

SUPPLEMENTARY MAP 8
Distribution map of *Sobralia gloriosa* Rchb. f.

SUPPLEMENTARY MAP 9
Distribution map of *Sobralia ruckeri* Linden & Rchb.f.

SUPPLEMENTARY MAP 10
Distribution map of *Sobralia tamboana* Dodson.

SUPPLEMENTARY MAP 11
Distribution map of *Sobralia hoppii* Schltr.

SUPPLEMENTARY MAP 12
Distribution map of *Sobralia pulcherrima* Garay.

SUPPLEMENTARY MAP 13
Distribution map of *Sobralia rosea* Poepp. & Endl.

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The effect of habitat transformation on a twig epiphytic orchid: Evidence from population dynamics

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The tropical Andean landscape has been dramatically transformed over the last century with remaining native forest limited to small fragments within a heterogeneous matrix of crops, cattle pastures, and urban environments. We aimed to explore the impact of habitat transformation on the population dynamics in an endemic twig epiphytic orchid located within the undisturbed forest and within modified matrix habitat in two regions with contrasting landscape structures: with a dominant shade coffee matrix and a dominant grassland matrix. Over 2 years, we surveyed 4,650 individuals of the Colombian endemic orchid, *Rodriguezia granadensis*. We undertook four post-breeding censuses in three sites in each region in both native forest and pasture sub-sites (12 sub-sites; 48 censuses in total), and constructed demographic transition matrices (n = 36). The transition probabilities were calculated using a Bayesian approach and population grow rates were evaluated using asymptotic models and elasticities using transient dynamics. Between regions, higher population growth rate and inertia (defined as the largest or smallest long-term population density with the same initial density distribution) was seen in the shade coffee-dominated landscape. Additionally, population growth rate and damping ratio was higher in forest compared with pasture, with lower convergence time for the forest subsites. These demographic patterns reveal the contrasting levels of population resilience of this orchid in different landscape structures with the more connected shade-coffee dominated landscape permitting some healthier populations with greater population growth and survival in forest than pasture. This study highlights that twig epiphyte colonization of isolated phorophytes in pastures should not be interpreted as a sign of a healthy population but as a temporal transitory period.

KEYWORDS

demography, landscape, matrix models, resiliency, *Rodriguezia granadensis*, tropical Andes, reproductive success, PPM

Introduction

Habitat fragmentation threatens the survival of populations and species in two main ways. Firstly, smaller, isolated populations in habitats with high fragmentation are more vulnerable to stochastic events (Fischer and Lindenmayer, 2007). These may be the result of environmental catastrophes, particularly in the context of increasingly extreme climatic events, random genetic processes, with the loss of evolutionary potential through genetic

drift (Lienert, 2004; Honnay and Jacquemyn, 2007) or demographic processes, with year-to-year variability in reproductive success (Tomimatsu and Ohara, 2010; Jacquemyn et al., 2012). Secondly, with the reduction in area of natural habitat, the abiotic conditions of the surrounding landscape change, with consequent negative impact on plant reproduction (Aguilar et al., 2006; Aguilar et al., 2019) and biotic interactions (Brosi, 2009; Briggs et al., 2013).

The impact of habitat transformation (Ritchie and Roser, 2013; Winkler et al., 2021) on ecological characteristics of species has been well-studied from diverse perspectives, such as life histories characteristics (Kolb and Diekmann, 2005; Bruna et al., 2009), extinction probabilities (Fréville et al., 2007), plant animal interactions (Benítez-Malvido and Arroyo-Rodríguez, 2008; Benitez-Malvido et al., 2016) and reproductive success (Brudvig et al., 2015; Vellend et al., 2017).

Following the theory of island biogeography, fragments of native habitat can be considered as islands within a "sea" of transformed terrain. In a mosaic of patches of different land use, the dominant, usually non-native background in a transformed landscape is known as the matrix (Fischer and Lindenmayer, 2007). Spatial matrix types, such as cattle pasture, different agricultural systems, or urbanization, have differential impacts on the connectivity between native habitat fragments, altering resource availability, as well as the activity of pollinators, seed dispersers, and herbivores (Jules and Shahani, 2003; Debinski, 2006).

For example, fragmentation and loss of habitat quality affect pollinator communities, including the so-called "orchid bees" (Apidae: Euglossini), impacting home ranges (Brosi, 2009) and reproductive success (Newman et al., 2013). Small, isolated plant populations are expected to have lower reproductive success when dependent on non-resident pollinators (Murren, 2002).

The tropical Andes represent a hotspot of biodiversity (Myers et al., 2000; Liang et al., 2022) and endemism (Gentry, 1982; Olson and Dinerstein, 1998) and at the same time is one of the geographical areas with the highest rate of anthropogenic habitat transformation (Etter and van Wyngaarden, 2000; Etter et al., 2006). These anthropogenic changes can influence demographic dynamics (Rodríguez-Echeverry and Leiton, 2021), survivorship or persistence of populations (Philpott et al., 2008). Nonetheless, our understanding of the impact of habitat transformation in this biodiverse region is limited (Hoang and Kanemoto, 2021; Winkler et al., 2021). In the neotropics, the influence of modified landscape mosaics on the diversity of birds, bats (Harvey and González Villalobos, 2007), insects (Vandermeer et al., 2019), and trees (Philpott et al., 2008) have been documented, however there are only a few studies focused on epiphytes (Richards et al., 2020), including epiphytic orchids (García-González and Riverón-Giró, 2013; Raventós et al., 2018), with only one study in the Andes (Parra Sánchez et al., 2016).

Epiphytic plants grow on the trunk, branches, twigs, and even the leaves (Alvarenga and Pôrto, 2007) of a plant host, the phorophyte, enabling growth in higher light conditions. Vascular epiphytes are one of the most dominant guilds of species in the tropics and are potentially highly impacted and endangered by habitat transformation (Hernández-Pérez and Solano, 2015; Osie et al., 2022). The distribution and survival of epiphytes is influenced by the landscape structure, phorophyte diversity, the age of the forest and tree size (Hietz, 1999).

The main plant families with epiphytic species are Bromeliaceae and Orchidaceae (Zotz, 2013). Some epiphytic species can also be rupiculous, growing on rock substrate, while others may also be terrestrial. Those species that are exclusively epiphytic are often limited to a particular ecological niche in a restricted zone of the architecture of the tree (Catling et al., 1986; Medeiros, 2010). The so-called twig epiphytes use as their substrate the smallest branch size, most often located in the outer fringe of the tree canopy (Ventre-Lespiaucq et al., 2017). Obligate twig epiphytes often are characterized by their accelerated life cycle, psigmoid or terete leaves, and thickened seed testa (Chase, 1987; Zotz, 2007).

Orchids specialized as twig epiphytes, while numerous, are phylogenetically restricted to Oncidiinae and Vandeae clade (Chase, 1987; Gravendeel et al., 2004). A limited amount of information of the life history of these species is available [*Tolumnia variegata* (Sw.) Braem, Calvo and Horvitz, 1990; Ackerman et al., 1996; *Erycina crista-galli* (Rchb.f.) N.H.Williams and M.W.Chase, Mondragón et al., 2007; *Ionopsis utricularioides* (Sw.) Lindl., García-González and Riverón-Giró, 2013] and most of the supposed advantages of being a twig epiphyte are circumstantial. It is commonly assumed that the advantage in being in the outer rim of the canopy twig epiphytes is the higher availability of light. However, this advantage may have tradeoffs (Ventre-Lespiaucq et al., 2017) including a greater risk of dehydration (Chase, 1987). A study in *T. variegata* found that plants located on twigs at the canopy edge had a reproductive disadvantage compared with those located within the tree canopy (Tremblay et al., 2010). Increased light availability may result in higher reproductive potential (flower production) but could also result in lower survival of the smaller individuals (for example because of desiccation), resulting in an overall decrease in the long-term persistence of the population.

Twig epiphytic orchids are often transitory pioneer species and frequently colonize phorophytes in transformed habitats. Given the tolerance of twig epiphytes to higher light intensities (Ventre-Lespiaucq et al., 2017), such populations on trees in transformed, open environment may be perceived to be as healthy as those in undisturbed forest habitat. However, this perspective may be misleading as the number of individuals may be temporary.

Our study aims to evaluate the impact of habitat transformation on the twig epiphytic species, *Rodriguezia granadensis* (Lindl) Rchb.f. This orchid is commonly distributed across Andean premontane and montane forests. Although endemic to Colombia, its natural tendency to colonize isolated phorophytes in open pastures is a major contributing factor to its classification of least concern (LC) in national red-list evaluations (Calderón-Sáenz, 2007; López-Gallego and Morales, 2021).

We used an approach which includes the complete life history of the species, following individuals in a mark-recapture approach and population projection matrices (PPM), comparing the demographic structure and dynamics in native forest fragments and on isolated phorophytes in pastures across two contrasting landscapes. We aim to understand the potential different demographic responses in each landscape and land use, thereby drawing inferences on the dynamics of orchid twig epiphyte populations in varying anthropogenically modified environments. We hope our findings may inform landscape management practices to promote orchid conservation in this biodiverse region.

As a null model, we would expect no differences in population dynamics between the two landscapes, a matrix dominated by either coffee crop or sugar cane and cattle grassland, nor between native forest and pastureland cover populations. If the main driver for the niche occupancy of twig epiphytes in the outer tree canopy is to maximize exposure to light, it could be expected that populations on isolated trees in an open environment such as pastures would present

more favorable demographic parameters, with a higher population growth rate and stability. Specifically, we aim to determine whether population dynamics of twig epiphytes are similar in native forest and pastures in the two regions across 2 years of survey considering the following parameters (1) deterministic population growth rate, (2) transient dynamics and (3) non-linear elasticity (transfer function) of the different life stages, (4) reproductive potential (fruit set), and (5) recruitment.

Materials and methods

Study species

Rodriguezia granadensis (Lindl.) Rchb.f. is widely distributed at mid-elevation (700–1,900 m.a.s.l.) in Andean Forest. This orchid frequently colonizes coffee or fruit tree plantations. The species is common and widely distributed and consequently an excellent model species to study the impact of changing landscapes in the northern Andes on epiphytic population dynamics (Ventre-Lespiaucq et al., 2017).

Rodriguezia granadensis has two flowering seasons a year (March–April and October–November), which coincide with bimodal peaks of rainfall (Calderón-Sáenz, 2007). It is pollinated by euglossine bee species–*Eulaema meriana* Oliver, *E. cingulata* Fabricius, and *Exaerete frontalis* Guérin-Méneville–that forage for nectar in a melitophilly syndrome behavior (Ospina-Calderón et al., 2015).

Study sites

Populations of *R. granadensis* were studied in three field sites each in two regions of the tropical Andes in Colombia, in the departments of Cauca, and Valle del Cauca (from here on "Valle"). These two regions, separated by approximately 150 km, are located at the same elevation (approximately 1,700 m.a.s.l.) on the eastern slope of western cordillera of the Andes (**Figure 1**).

The Cauca region is in the Colombian massif of the Popayán plateau with sun and shade coffee crops in an agroforestry mosaic, with mixed and forestry crops, small fragments of forest and riparian forests (Criollo and Bastidas, 2011; Arenas-Clavijo and Armbrecht, 2018). Although the coffee landscape is increasing in agricultural intensity, to the detriment of biodiversity (Armbrecht, 2003; Philpott et al., 2008; Harvey et al., 2021), it continues to host more diversity as an agroecosystem (Letourneau et al., 2011) than extensive monocultures such as the sugar cane and cattle ranching model in the Valle del Cauca department to the north (Marull et al., 2018; Sardi et al., 2018). In the latter, we find a few isolated forests in a predominantly pasture matrix, where the landscape and biodiversity has been dramatically affected (Torres et al., 2012; Vélez-Torres et al., 2019).

The southern region in Cauca, has a mean annual precipitation of ± 2,120 mm, and temperature of 15°C (IDEAM, 2010; Puertas-Orozco et al., 2011). The three field sites in this region were: (1) Calibío (Cl) (2° 37.446′ N, 76° 33.525′ W); (2) Cajibío (Cj) (2° 38.888′ N, 76° 32.328′ W); and (3) Piendamó (Pi) (2° 41.126′ N, 76° 33.710′ W). The region to the north, in Valle del Cauca, has a mean annual precipitation ± 1,480 mm and temperature of 18°C (IDEAM, 2010; Puertas-Orozco et al., 2011). Our three field sites were (1) Hondonada (H) (3° 49.896′ N, 76° 26.043′ W), (2) Lilas (L) (3° 50.986′ N, 76° 26.344′ W) and (3) the National Forest Reserve of Yotoco (Y) (3° 52.712′ N, 76° 26.291′ W). The three field sites within each region had pairwise geographic distances between 5 and 15 km (**Figure 1**).

Survey

At each of the six field sites we surveyed plants of *R. granadensis* in two sub-sites of contrasting land cover: native forest (continuous canopy) and pasture (grassland with isolated trees), for a total of 12 sampled sub-sites. From here on, we refer to three different analysis levels: **Region** comparing Cauca with Valle, **Site** Calibío (Cl), Cajibío (Cj), Piendamó (Pi) in Cauca, and Hondonada (H), Lilas (Li), and Yotoco (Y) in Valle and **Sub-site**, contrasting landcover, forest or pasture. Within sub-sites all individual orchids present in each phorophyte (host tree) were marked and counted until reaching 300 at the first census. The position of each phorophyte sampled was registered with a GPS Global Positional System (Garmin Oregon 750), and the minimum convex polygon for each sub-site was calculated to report phorophyte distance and density (QGIS 3.26). Individual orchid plants were marked with permanent Dymo tags for monitoring over consecutive censuses.

While density per meter square is a common metric used to describe the dispersion pattern of many plant species, it is not always an adequate description of the dispersion pattern of epiphytic orchids (Tremblay, 1997). Because epiphytic orchids are dependent on the presence of the host tree, measuring density per host tree is a more realistic index, and we took both of these variables into account.

We surveyed plants at four different times, resulting in three transition matrices between consecutive post-breeding censuses for each site. In the first census in March 2017, we tagged the first plants in each sub-site. For the subsequent three censuses (Oct. 2017, March 2018, and Oct. 2018) when additional individuals were detected, these were tagged too and included in the population analyses. Thus, an additional 30 to 50 plants were registered per sub-site per survey for a total of between 348 and 440 plants per sub-site and a grand total of 4,650 unique individual plants in the study (**Supplementary Table 1**).

For each plant we registered the number of live pseudobulbs, inflorescences, flowers, and fruits as an index of reproductive potential in addition to survival among time periods. The reproductive potential for a specific stage was estimated as the number of fruits/number of flowers in the time period (Sabat and Ackerman, 1996). The expected number of recruits at time t is assumed to be proportional to the fruit set at time t-1, consequently, recruitment does not include the seed stage or dormancy of seeds (Tremblay and Hutchings, 2002).

Data analysis

We reviewed the distribution of all the demographic and reproductive variables per region, site, and subsite. After conducting assumption tests with Shapiro Wilks and without transformation, we ran an analysis of variance ANOVA to test for differences in demography and reproductive variables between region and site. For subsites, we ran a paired t-test.

For the population projection matrices (PPM), we applied the life cycle structure previously determined for this species, based on

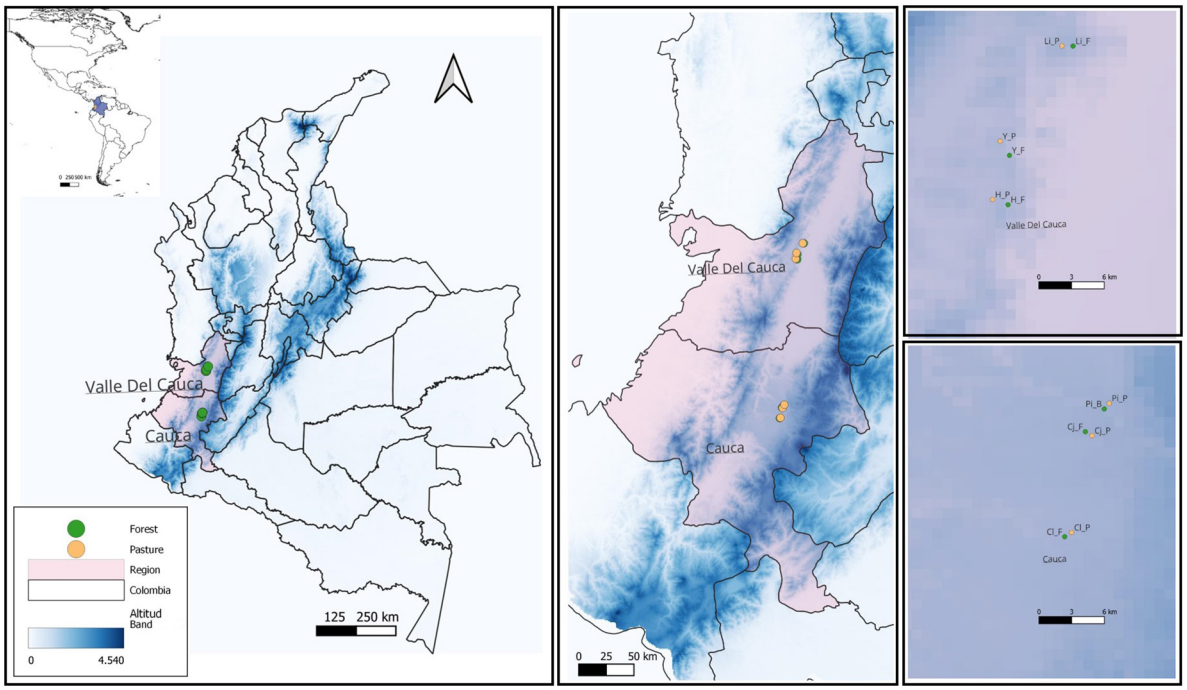

FIGURE 1
Geographical location study sites and experimental design showing sampling organization over three scales, Region: Cauca and Valle. Site: Cl, Calibío; Cj, Cajibío; Pi, Piendamó; H, Hondonada; Li, Lilas; Y, Yotoco. Sub-site: F, forest; P, pasture.

pseudobulb and inflorescence number (Ospina-Calderón, 2009), and the methodology for PPM developed for orchids, following Tremblay and Hutchings (2002) and Martorell et al. (2022). Our simplified life history of *R. granadensis* is based on four size classes describing the life stages: (1) Seedling (S), individuals lacking pseudobulbs; (2) Juvenile (J) with 1–2 pseudobulbs; (3) Small adults–stage 1 (A1), possessing 3–6 pseudobulbs and no more than one inflorescence; and 4. Large adults–stage 2 (A2) for plants possessing more than 7 pseudobulbs with one or more inflorescences. Transitions between life stages from one census to the next were recorded as growth (G); fecundity (Fe), stasis within the same life stage (L); and reversal (R) (**Figure 2**).

Estimating transition probabilities

From the data registered during the four censuses (March and October, 2017 and 2018) we calculated parameters for 36 transition matrices (time period x sub-sites). Each matrix corresponds to a time period: Time 1–March to October 2017; Time 2–October 2017 to March 2018; Time 3–March to October 2018. Thus, three matrices for each of the 12 sub-sites were constructed. The transition probabilities were estimated using a Bayesian approach (Tremblay et al., 2021). This analysis is more appropriate for the current dataset for two reasons. Firstly, it resolves issues for estimating the parameters of some transitions with small sample sizes (for example, seedlings were scarce or not detected in some populations). Secondly, the parameter estimates (transitions, survival, death, and stasis) follow the required beta distribution and the credible intervals are bounded between 0 and 1. With this Bayesian approach infrequent transitions can be estimated while avoiding improbable values that may be generated with small sample sizes or few observed transitions for some of the specific stages (Tremblay et al., 2021). For the Bayesian analysis, prior data for the matrix (**Table 1**) were selected from a previous census undertaken in the population of Yotoco in 2008 (Ospina-Calderón, 2009) with an effective sample size of $n = 1$. Consequently, this weak prior has little impact on the transition probabilities when sample sizes are large. This choice of an effective sample size yields posterior parameters that are dominated by the data.

Population growth rates

We employed population projection matrix (PPM) analysis to evaluate the asymptotic populations growth rate, lambda. When lambda is equal to one (including the credible intervals, CrI) populations are considered to be stable, while lambda values either smaller or larger than one (with the CrI) indicate a decreasing or increasing population size, respectively. The median population growth rate and the CrI were calculated with 15,000 simulations corresponding to the posterior lambda values and the CrI (Tremblay et al., 2021).

Transient dynamics, transfer function

Transient dynamics analysis and the indices described by Stott et al. (2012a) are mathematical approaches to study the short-term effect of ecological disturbances or perturbation on the population structure of a species in addition to understanding the impact of population structure not at equilibrium (Stott et al., 2011). This innovative approach to understanding short–term dynamics has been applied broadly in plants (McDonald et al., 2016), for example, plant

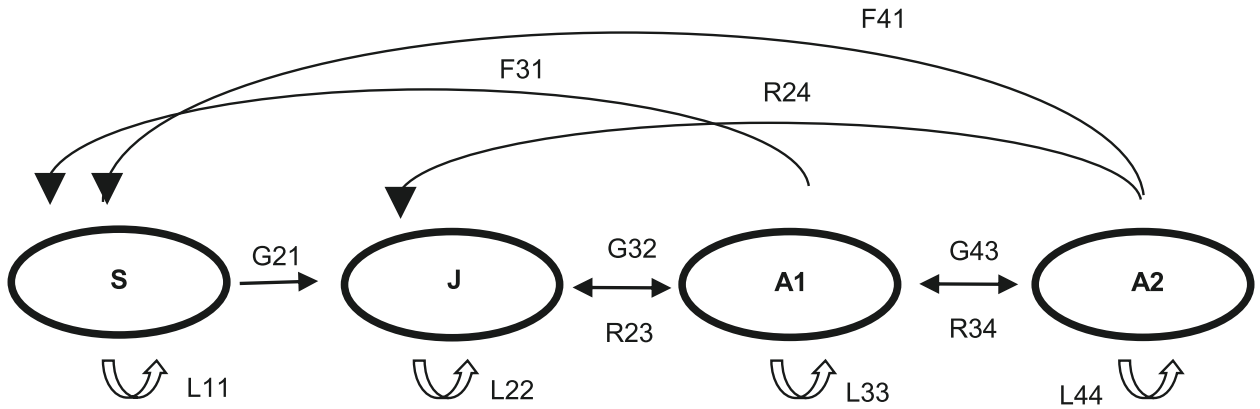

FIGURE 2
Life cycle diagram of *Rodriguezia granadensis*. S, seedling; J, juvenile; A1, adult, stage 1; A2, adult, stage 2. Transitions: G, growth; F, fecundity; L, stasis in the same stage; R, reverse.

invasions (Iles et al., 2016) and orchids (Raventós et al., 2015; Ortiz-Rodríguez et al., 2020). Transient dynamics PPM models are time-invariant, however by varying the starting demographic distribution, and modeling demographic stochasticity whether of biotic, abiotic, and anthropogenic origin, transient dynamics may result in a stage distribution that differs from the stable stage distribution.

The different starting scenarios lead to either a short-term increase in population size and density (*amplifications*) or a short-term decrease (*attenuation*). If no other perturbations or disturbances are present, then the transient dynamics models are expected to stabilize to the stable stage distribution. The time to reach the stable stage is the transient period (Stott et al., 2011). One of the most useful measurements of transient population density and growth are reactivity and inertia. These indices and their bounds describe the majority of variation in transient population density with biological interpretations because they describe shorts term changes (Stott et al., 2011). In general, orchid populations are not at stable stage distribution (Schödelbauerová et al., 2010; Tremblay et al., 2015), however a comprehensive review is still lacking.

Transfer function is an approach for evaluating the non-linear effect of perturbation on population dynamics. The traditional approach has been to evaluate the elasticities of the parameters of the matrix (Caswell, 2000), with the limitation that elasticities are assumed to be linear and consequently are usually more applicable when perturbation is small (Stott et al., 2012a). The advantage of transfer function analysis is that it can elucidate the possible impact across a wider range of perturbation without assuming that the response is linear.

Software

All the analysis was performed in the R 4.2.0 environment. The PPM parameters based on a Bayesian approach were evaluated using the *raretrans* package (Tremblay et al., 2021). The asymptotic population growth rates, transient dynamics, and transfer function were attained using the *popdemo* R package (Stott et al., 2012b) using the posterior matrices. Data were visualized, contrasted, and wrangled using the *ggplot2* and tidyverse packages (Wickham, 2016; Wickham et al., 2019).

TABLE 1 Priors for the transition matrix for estimating the posterior transition probabilities.

Stages	*S*	*J*	A1	A2
S	0.30	0	0	0
J	0.09	0.35	0.001	0
A1	0.01	0.10	0.60	0.06
A2	0	0.05	0.07	0.84

Data from a previous study on *Rodriguezia granadensis* and Yotoco population (Ospina-Calderón, 2009). S, seedling; J, juvenile; A1, adult, stage 1; A2, adult, stage 2.

Results

We surveyed a total of 4,650 plants, in 12 sub-sites of both native forest and pasture land cover sub-sites in three sites each in two regions with differing landscape composition: shade-coffee dominated, and pasture-dominated (**Supplementary Table 1**). A total of 1,636 individuals died across the survey period (**Supplementary Table 2**).

Spatial distribution and fruit set

The spatial distribution of *R. granadensis* plants and phorophytes varied among landcover sub-sites in a similar way in both regions. In the native forest populations, plants were found over areas from 1,830 to 6,218 m^2, while the number of phorophytes varied from nine to 96. The number of orchids per phorophyte varied between four and 10 in each forest sub-site. In the pasture sub-sites in both regions, the distribution areas were half to five times less (582 to 1,127 m^2) with a range of nine to 35 phorophytes and between 10 and 46 individuals per phorophyte (**Supplementary Table 1**). Thus, population density was 2 ind./m^2 (sd = 0.51) in forest sub-sites, and 10 ind./m^2 (sd = 5.16) in pasture sub-sites. A more aggregated distribution in the isolated phorophytes in the pasture matrix was observed. The number of plants per phorophyte were significantly lower in forest sub-sites with fewer plants per tree in forest (6.93, ANOVA sd = 2.22, $p = 0.01$) than in pastures (25.67, sd = 13.95).

The average fruit set was 0.055 fruits/flowers (sd = 0.035) for all sub-sites and seasons (**Supplementary Table 3**). However, over

TABLE 2 Summary of *Rodriguezia granadensis* fruit set for forest (F) and pasture (P) sub-sites in two regions, Cauca (C) and Valle (V), Colombia.

Region	Sub-site	Fruit set	Sd	Mean number of fruits	Number of fruits	Number of flowers
C	F	0.0733	0.0464	18.58	223	3151
V	F	0.0625	0.0362	13.75	165	2861
C	P	0.0383	0.0248	12.50	150	4733
V	P	0.0433	0.0235	15.58	187	3841

Mean and total number of fruits over four censuses in 3 sub-sites of each land cover type in each region. Sd, standard deviation.

both regions, fruit set was significantly greater for forest sub-sites compared with pasture sub-sites (Forest mean = 0.067, sd = 0.041; Pasture mean = 0.040, ANOVA sd = 0.023, $p = 0.01$) (Table 2). The exception was in the Hondonada pasture (Valle), which presented a greater number of fruits than the forest (Supplementary Table 4).

Population projection matrices (PPM), asymptotic population grow rate

A total of 36 transition matrices representing three time periods for each of the 12 subsites were constructed: three subsites each of forest or pasture within each of the two regions Cauca and Valle. The most common transition detected for all stages was for stasis (L), with plants remaining in the same stage through at least two consecutive censuses (Figure 2). Over both regions, in forest sub-sites the most common transition was for the Adult 1 stasis, L33, and in pasture sub-sites for Adult 2 stasis, L44 (Supplementary Table 2).

Overall, the intrinsic population growth rates (lambda) in all 36 matrices ranged from a minimum of 0.742 to a maximum of 1.268 (Figure 3; Supplementary Table 5). A striking difference was seen between the forest and pasture subsites over both regions. In the forest sub-sites 12 of the 18 PPM yielded a lambda greater than one (increasing population), with two less than one. In contrast, in the pasture subsites, 12 PPM yielded a lambda less than one, with two being greater than one. The distribution of population reduction, stability and growth was not equal among the forest sub-sites (Fisher's exact test = 12.42, df = 2, $p = 0.002$), however it was independent of regions, although forest populations may be of slightly better health in both Cauca and Valle.

Among sub-sites and time periods, variation was seen in the population growth rates, from reductions of close to 30% to increases of 25%. The sub-site with the largest population reduction was at Cajibío pasture, in Cauca (Time 2, lambda 0.742; 95% CrI 0.667–0.819). The two sub-sites which had the largest increase were in forest, in Valle, Hondonada (Time 1, lambda 1.268, 95%CrI 1.187–1.349) and at Cauca, Calibío (Time 1, lambda 1.199; 95%CrI 1.147–1.253).

Transient dynamics

The transient dynamics indices revealed that the convergence times to stable structure tended to be smaller in forest than in pasture sub-sites for all sites, with the exception of Cajibío in the coffee-dominated landscape in Cauca. The shadow diagram confirms that Cj Forest is more likely to grow than pasture, with darker zones showing decline and tendency to extinction in 5 to 10 years (Figure 4; Supplementary Figure 1). Additionally, higher values for inertia, reactivity and damping ratio indicate greater resilience for forest than pasture sub-sites (Supplementary Table 5).

The upper value for inertia upper was for the forest subsites in the sites Calibío, Cl (Cauca) and Hondonada, H (Valle) and for inertia low, the lowest was Calibío pasture and Lilas, Li (Valle) pasture. Reactivity confirms this pattern of more resilient plants in the forest sub-sites, with a higher register for Calibío forest and lower for Lilas (Valle) pasture (Supplementary Table 5).

Transient population dynamics simulation for 50 flowering seasons (25 years) revealed a greater tendency for populations to decline and possible extinction in 5 to 10 years in pasture compared with forest populations (Figure 5; Supplementary Figure 2). In four of the six pasture sub-sites over both regions, simulations indicated probable population decline tending to extinction in 5 to 10 years (Supplementary Figure 2). In contrast, for the forest sub-sites, these simulations suggested population growth for four of the six sub-sites, with only Cajibío (Cauca) and Lilas (Valle) indicating a decrease in population size.

Perturbation analysis

Perturbation analysis using the non-linear elasticities approach, transfer function, revealed a non-linear relation on relative importance of the influence of perturbation for each stage (Supplementary Figure 3). It is evident that perturbation results in non-linear response of population growth rate as a function of the amount of perturbation in almost all of the parameters. This is most evident in the stasis stage (the diagonal of the matrix) where most have a narrow peak with a rapid decrease and increase around an optimum. Increases in reproductive success (fruits/flowers) show a near linear response in almost all cases. While transitions to the next life stage results in a "U" shape response in some cases, the pattern is inconsistent across sites and time periods showing how the population is likely to respond if there is an increase or decrease in the parameters and how that would affect growth rate.

Discussion

This is the first study to compare the demographic patterns of a twig epiphytic orchid between populations in native and transformed habitat matrices in the tropical Andes. Our findings of less favorable population dynamics for *R. granadensis* in transformed compared with forest land covers, and between two contrasting landscape structures has important implications for the evaluation of the conservation status of this species and will inform landscape management practices to promote conservation of other similar twig epiphytic orchids in this biodiverse region.

Previous studies of tropical epiphytic communities have shown that species diversity and abundance decrease over gradients of increasing human impact (Larrea and Werner, 2010; Hylander and Nemomissa, 2017). However, in the present study, the populations of *R. granadensis* colonizing isolated fruit or shade trees within a transformed pasture matrix had a higher density of plants per phorophyte compared with the forest sub-sites (Supplement Table 1). This may be partly explained as isolated trees in open pastures tend to grow larger and wider crowns (Elias et al., 2021).

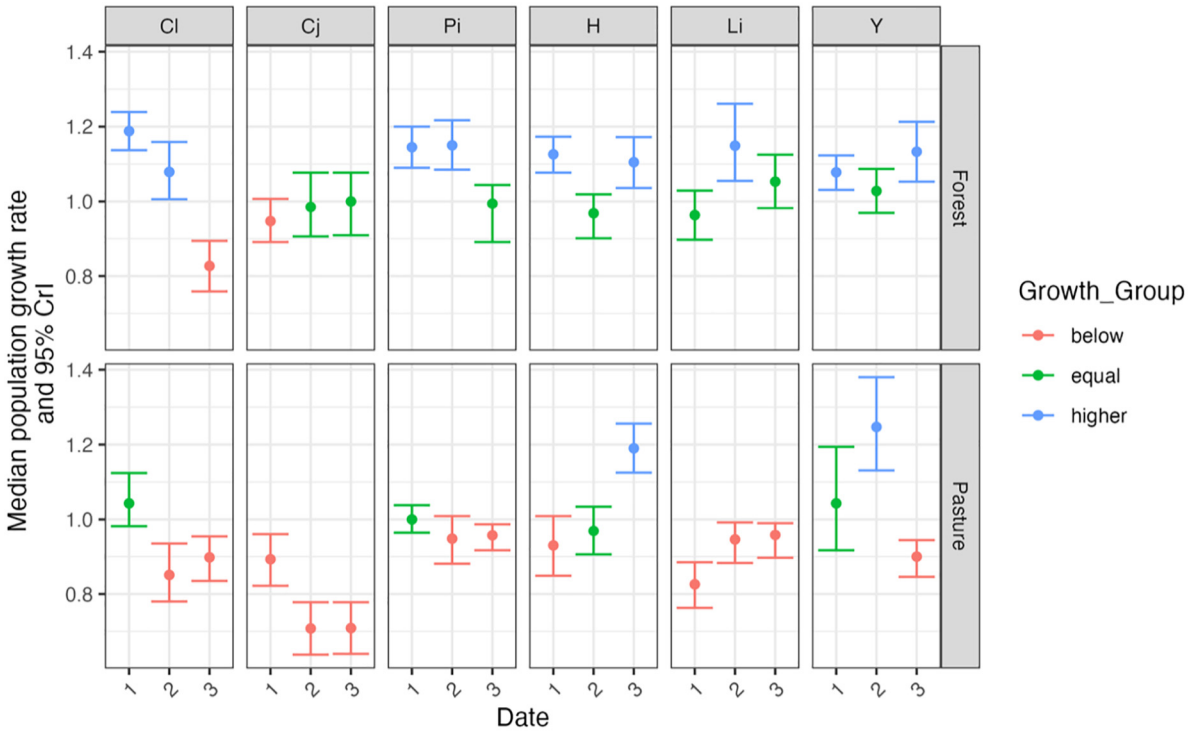

FIGURE 3
Median posterior asymptotic population growth rate and 95% credible intervals estimated for *Rodriguezia granadensis* population at two subsites, Forest and Pasture in six Sites (Cauca sites: Cl, Calibío; Cj, Cajibío; Pi, Piendamó. Valle sites: H, Hondonada; Li, Lilas; Y, Yotoco), over three time periods; Time 1, Mar.–Oct. 2017; Time 2, Mar. 2017–Oct. 2018; Time 3, Mar.–Oct. 2018. Red are populations/time periods where lambda was significantly smaller than 1, green for lambda equal to one (not significantly different from stability), and blue for lambda significantly larger than one.

In Andean human-transformed landscape, Köster et al. (2011) found that tree traits explain 60% of the epiphytic community composition in an Ecuadorian cloud forest, where the isolated trees act as steppingstones that permit some persistence of epiphytes outside of the forest in a changing landscape mosaic (Köster et al., 2009; Elias et al., 2021). In these circumstances some species become denser and more abundant outside of the forest, possibly as a response to scarce available of phorophytes (Larrea and Werner, 2010), as well as changing abiotic conditions.

Nonetheless, studies in other epiphytic species have shown that in transformed habitat, population density initially increases quickly, only to later decrease, often leading to extinction, depending on time and distance to the forest source of seeds (Pellegrino et al., 2015; Hylander and Nemomissa, 2017). Thus, the high-density populations of *R. granadensis* in open pasture found in this study may be of a transient nature. Continued population monitoring over a longer period is needed to gauge this temporal effect.

The abundance of reproductive adults and fruit set was greater in the forest environment (**Supplementary Table 3**). Native forests likely comprise a more suitable ecological niche and adequate pollinator community compared to isolated trees within a pasture matrix. With increasing isolation of phorophytes from the native forests, a reduction in the number of Euglossine pollinators visiting these isolated patches has been observed (Briggs et al., 2013).

Our study reveals that the demographic health of orchid twig epiphytes is negatively influenced in transformed environments; hence, in both landscape structures, the forest sub-sites showed higher asymptotic population growth rate with greater resilience (inertia, reactivity) and a lower short-term population decline. Population growth simulation across multiple time periods suggests that the forest populations are less likely to go extinct as compared to pasture sub-sites. The likelihood of extinction of forest sites within a 20-year period is 20% (lambda mean 1.021), while pasture sites have a 45% (lambda mean 0.938) probability of extinction (**Figure 2**; **Supplementary Table 5**). According to Criterion C for the IUCN Red List evaluations, a species may be categorized as vulnerable with less than 1,000 mature individuals in each subpopulation and/or a probability of extinction of 10% in 10 years. While it is likely that *R. granadensis* has more than a total of 10,000 individuals across its range (the central aspect of criterion C), our findings indicate that the likelihood of subpopulation extinction is high even in the forest environment.

Only a small number of multi-period censuses of orchid population dynamics have been undertaken in the tropics, and these have similarly found a negative impact on orchid populations in landscapes with anthropogenic activity. In a study of three epiphytic species growing on coffee trees, *Oncidium poikilostalix* (Kraenzl.) M.W.Chase and N.H.Williams, *Lepanthes acuminata* Schltr. and *Telipogon helleri* (L.O.Williams) N.H.Williams and Dressler in Chiapas México, lambda was greater in populations in unmanaged coffee plantations compared with managed plantations (García-González et al., 2017; Raventós et al., 2018). In the terrestrial tropical invasive species, *Oeceoclades maculata* (Lindl.) Lindl. a higher population growth rate was noted within a Mexican forest than in a managed coffee plantation (Riverón-Giró et al., 2019). While in *Phaius flavus* (Blume) Lindl. in southeast China, Li et al. (2022) found that populations tended to decrease, and this change was attributed to the low germination rate in the wild and the

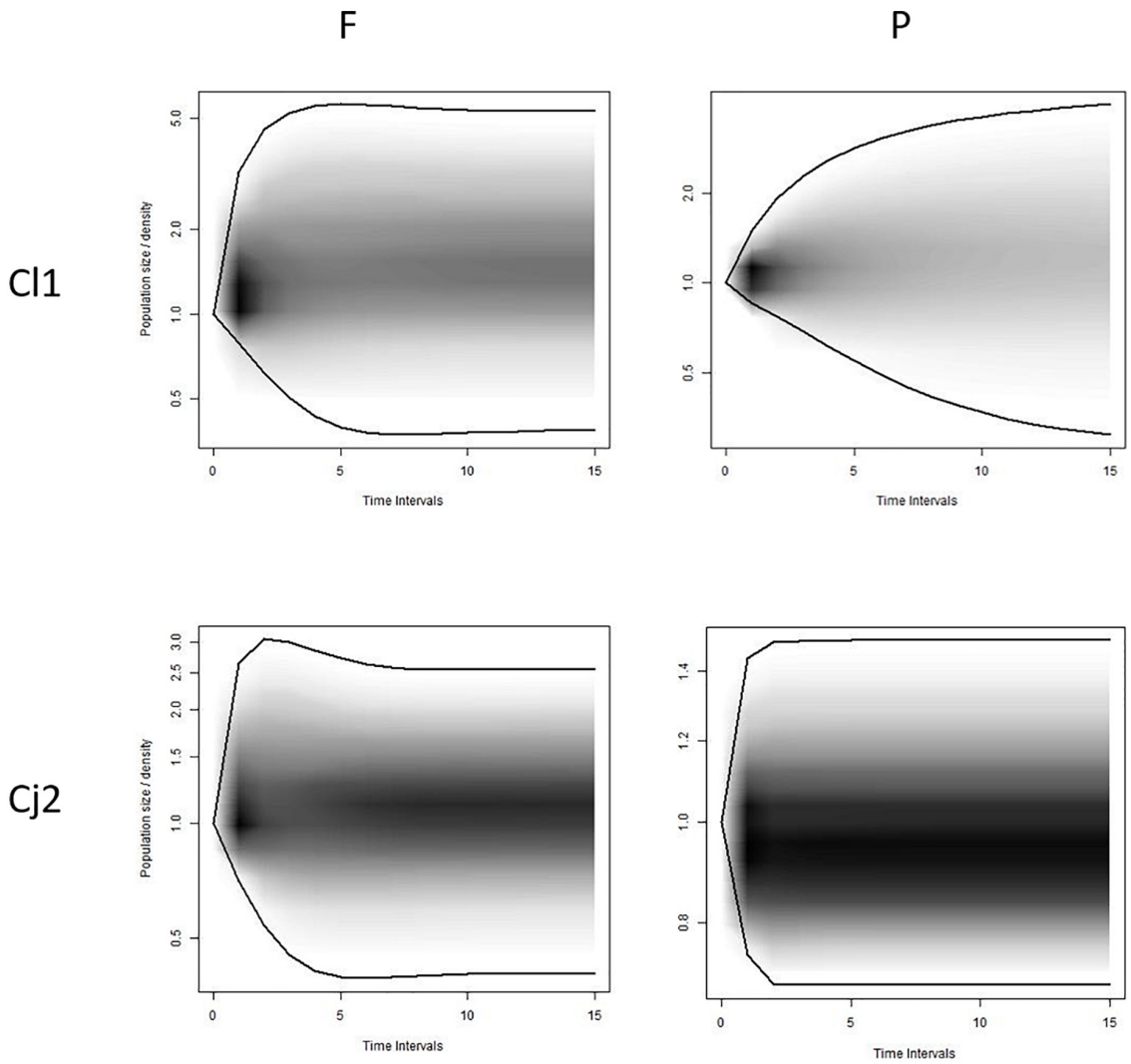

FIGURE 4
Visualizations of transient dynamics for four selected sub-sites at different time periods showing the diversity of plausible transient responses. Data from; Cl1, Calibío Time period 1; Cj2, Cajibío Time period 2, (F) Forest, (P) Pasture. Decreasing Cj2P, stable, Cl1F and increasing Cj2F. y Cl1P the figure represents the diversity of transient dynamics as a function of the initial starting vector (the number of distribution of individuals at each stage). Darker shading indicates a higher probability of the population size in that range.

loss of adult individuals caused by anthropogenic disturbances. In this current study, individual plants tended to remain in the same stage from one census to the next (**Supplementary Table 2**). Such stasis as the predominant life history process has also been registered in other neotropical epiphytic orchids (Tremblay and Hutchings, 2002; Crain et al., 2019). In general, in iteroparous forest plants with long lifespans, multi-year reproductive adult stages, and generation overlap, populations often consist of a preponderance of adults of varying sizes that remain in the same stage and contribute to population recruitment through the reproductive (Fe) stage (Silvertown et al., 1996). In contrast, iteroparous plants in open habitat plants typically exhibit populations with predominantly growth (G) and reproductive (Fe) transitions (Silvertown et al., 1993; Franco and Silvertown, 2004). Our data show that *R. granadensis* populations in an open habitat retain the forest strategy, with persistence of adults, lower generational turnover, fewer seedlings and juveniles that survive to adulthood, slower growth rates, all leading to declining populations.

Population convergence time to a stable state distribution was lower for forest sites than in pastures, which suggest that forest habitat may be beneficial for population stability and promoting higher population resiliency (**Supplementary Table 5**). Furthermore, values for inertia and reactivity, amplification and attenuation had wider intervals for forest populations, and so greater resilience in the face of changing environmental circumstances, including habitat transformations or climate change. Rapid fluctuations in the population size through time (**Figure 4**) could be advantageous if a population can increase rapidly after a size reduction due to stochastic phenomena. However, it may also suggest vulnerability if the fluctuation results in a rapid decrease in population size, as noted in *Lepanthes caritensis* Tremblay and Ackerman, *Dendrophylax lindenii* (Lindl.) Benth. ex Rolfe, *Broughtonia cubensis* Cogn (Raventós et al., 2015,b; Tremblay et al., 2015; Crain et al., 2019).

Most of the transient dynamics simulations for *R. granadensis* reflect the tendency for rapid reduction and high probability of extinction in about five to 10 years. Although some subsites

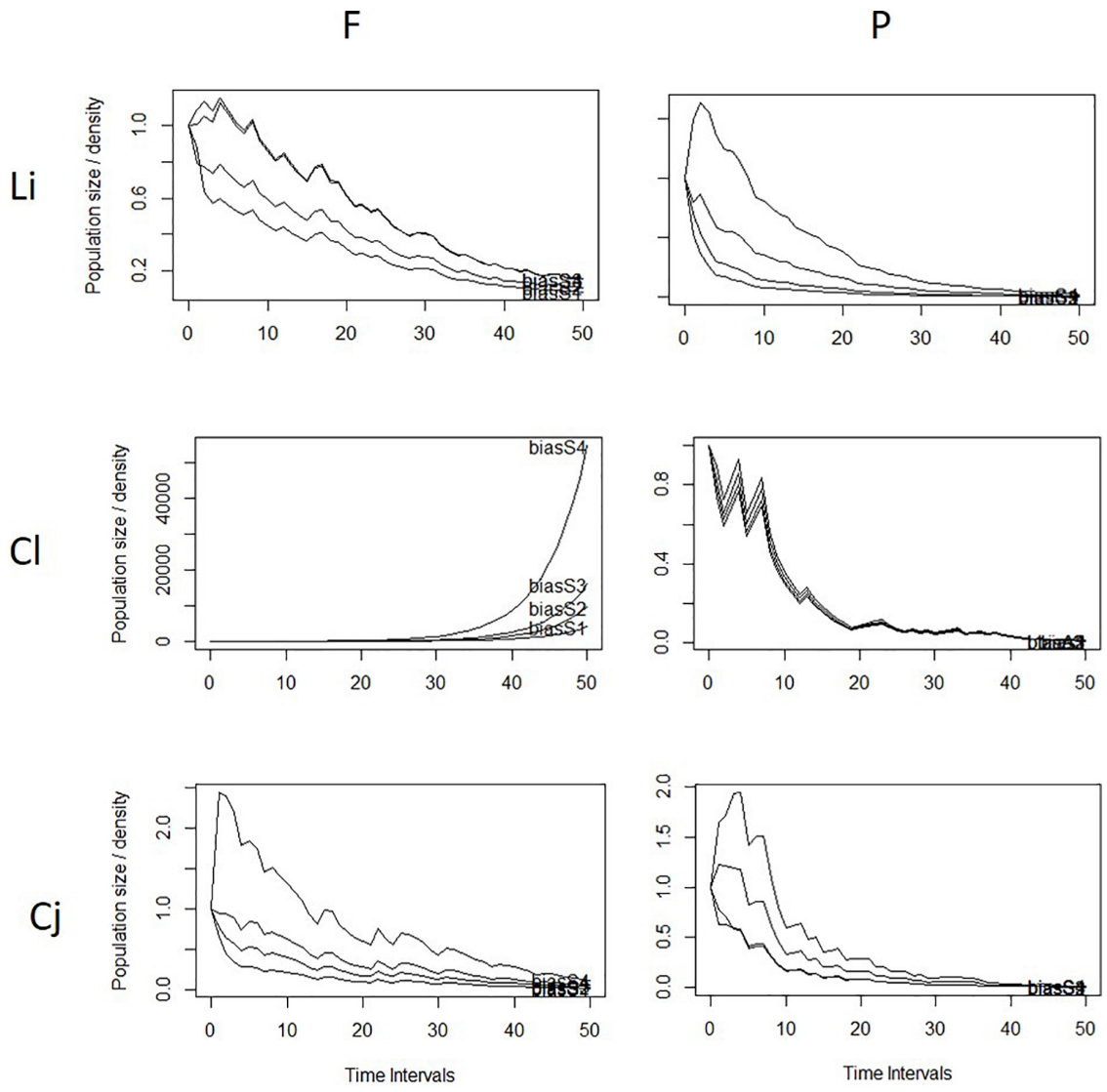

FIGURE 5
Rodriguezia granadensis transient dynamic simulation for 50 seasons, 25 years for tree of six study sites, Li, Lilas; Cl, Calibío; Cj, Cajibío. Subsites: F, forest; P, pasture. Bias S1, S2, S3, S4, stage-biased demographic vectors.

showed population growth (Cl, Pi, H, Y forest and Pi, H pastures), the remaining subsites were near "equilibrium" without increased tendency to growth for more than 10 years. Even though populations are near equilibrium this does not necessarily guarantee that these populations will persist. A number of studies have shown that even when population sizes fluctuate, they are vulnerable to extinction when stochastic events are common (Raventós et al., 2015b; Crain et al., 2019). Twig epiphytes may be highly vulnerable to stochastic events, as loss of small branches as a consequence of the architectural growth of trees and competition with surrounding trees may result in reduced niche availability for these obligate small branch epiphytes.

Inherent fluctuations of epiphytic and twig epiphytic habit represent important constrictions for population growth, structure and distribution of *R. granadensis*. Transient dynamics are highly influenced by the initial vector and therefore linked to explosion or extinction and stochastic phenomena (García-González et al., 2017; Raventós et al., 2018). The pattern and intensity of fluctuation in population size may be exacerbated by natural phenomena. For example, in Central American and Caribbean orchid populations, the growth rate and high intensity fluctuations are mediated by stochastic disturbance due to large storms or hurricanes (Crain et al., 2019; Ortiz-Rodríguez et al., 2020; Raventós et al., 2021).

Perturbation analysis allows us to identify the effects of probable changes in each transition on the growth rate (Stott et al., 2011). In *R. granadensis* the stasis stages may be the most elastic as small changes the parameters could result in large, non-linear changes in population growth rates, most often showing a pattern close to a narrow inverted "U."

Our analyses show that *R. granadensis* populations have lower survival probability when colonizing phorophytes dispersed in a pasture matrix as compared to forest sites. The diminished persistence of this orchid in a modified landscape can likely be considered an extinction debt. Colonization of isolated trees may prevent extinction in the short term, but the persistence of these sites may depend on the dynamics of the sink-source and the distance from a more suitable forest fragment (Pellegrino et al., 2015;

Hylander and Nemomissa, 2017). While orchid populations within the forest and pasture landscape may function as a sink-source dynamic, the importance of the source vs. sink processes are presently little understood. Apposite questions include: how important is this process for the persistence of pasture populations, and is the direction of the seed source always from forest to pastures sites? Moreover, stochastic processes linked with the natural population dynamics of the species are drivers of epiphyte presence and persistence, and subsequently their interaction in land transformation and habitat fragmentation need to be considered (Armbrecht, 2003; Rivera-Pedroza et al., 2019; Zewdie et al., 2022).

Conclusion

The endemic twig epiphyte, *R. granadensis,* is present in anthropogenically-transformed land covers, but analysis of asymptotic and transitory dynamics indicates that these populations have lower viability than those in native forest fragments. Populations on isolated trees have lower generational turnover, fewer seedlings and juveniles that survive to adulthood, slower growth rates, and, in general, declining populations. Our data suggests that the demographic dynamics of epiphytic orchids are of a fluctuating nature, which makes them more vulnerable to disturbances and stochastic events. Since *R. granadensis* is a species categorized as of least concern (LC) according to IUCN Red List criteria, a more hopeful pattern in its population dynamics was expected, especially since it is found more or less frequently in disturbed landscapes. This contradiction in the health of a species when comparing observed long-term population growth rates and IUCN criteria may in part be that IUCN criteria used are those which do not explicitly include the ecology and long-term dynamics of the species but a snapshot of the population based on multiple assumptions which may not be predictors of the future health for some species.

Data availability statement

The original contributions presented in this study are included in this article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Author contributions

NO-C collected the data and ran the analysis. All authors contributed equally on writing and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1135316/full#supplementary-material

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Floral and genetic divergence across environmental gradients is moderated by inter-population gene flow in *Platanthera dilatata* (Orchidaceae)

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Understanding how natural selection acts on intraspecific variation to bring about phenotypic divergence is critical to understanding processes of evolutionary diversification. The orchid family is well known for pollinator-mediated selection of floral phenotypes operating among species and along environmental or geographic gradients. Its effectiveness at small spatial scales is less understood, making the geographic scale at which intraspecific floral variation is examined important to evaluating causes of phenotypic divergence. In this study, we quantified phenotypic variation in the orchid *Platanthera dilatata* across 26 populations in coastal Southeast Alaska and compared this to edaphic and genetic variation at microsatellite loci. We sought to determine (1) if flower morphological variation is structured at smaller geographic scales, (2) the extent of genetic divergence in relation to phenotypic divergence, (3) the scale at which inter-population gene flow occurs, and (4) the relative importance of geographic distance and abiotic factors on population genetic structure. Two morphological groups were found to separate based on lip and spur length and are restricted to different habitats. Small-flowered forms occur in muskeg bogs, whereas large-flowered forms occur in fens and meadows, and rarely in sub-alpine habitat. Genetic analyses were concordant with the morphological clusters, except for four small-flowered populations that were genetically indistinguishable from large-flowered populations and considered to be introgressed. In fact, most populations exhibited some admixture, indicating incomplete reproductive isolation between the flower forms. Pollinators may partition phenotypes but also facilitate gene flow because short-tongued Noctuidae moths pollinate both phenotypes, but longer-tongued hawkmoths were only observed pollinating the large-flowered phenotype, which may strengthen phenotypic divergence. Nevertheless, pollinator movement between habitats could have lasting effects on neutral genetic variation. At this small spatial scale, population genetic structure is only associated with environmental distance, likely due to extensive seed and pollinator movement. While this study corroborates previous findings of cryptic genetic lineages and phenotypic divergence in *P. dilatata*, the small scale of examination provided greater understanding of the factors that may underlie divergence.

KEYWORDS

cryptic divergence, flower variation, genetic structure, gene flow, soils, isolation by distance, isolation by environment, *Platanthera dilatata*

1. Introduction

Understanding the origin and maintenance of intraspecific variation is of central importance to evolutionary biology because they inform our understanding of diversification across space and time and illuminate the process of speciation (Pinheiro et al., 2018). Substantial intraspecific phenotypic variation may indicate the maintenance of polymorphisms over otherwise connected populations (Nobarinezhad and Wallace, 2022), or it could indicate the presence of evolutionarily divergent cryptic lineages that exhibit parallel ecological responses (Kahl et al., 2021). Whereas a polymorphic species is expected to experience spatially and temporally heterogeneous gene flow among populations, cryptic lineages exhibiting genetic divergence should be isolated from one another (Surveswaran et al., 2018). While genetic tools have been especially useful for identifying cryptic lineages, integrated approaches involving multiple data types and widespread sampling of populations provide not only the identification of cryptic lineages but also clues about their divergence and geographic spread (Surveswaran et al., 2018; Liu et al., 2022). When examined deeply, many species have been found to comprise cryptic lineages (Pinheiro et al., 2018). Linking such divergence with pollinator selection of floral traits is critical to understanding how it integrates with co-evolutionary processes in determining ecological speciation (Van der Niet et al., 2014).

The orchid genus *Platanthera* (L.) Rich. contains many phenotypically polymorphic species (e.g., Robertson and Wyatt, 1990; Wallace, 2003a; Bateman and Sexton, 2008; Bateman et al., 2013; Adhikari and Wallace, 2014) and potentially cryptic lineages (Wettewa et al., 2020). As in many orchids, this phenotypic variation is frequently attributed to pollinator-mediated selection (Hapeman and Inoue, 1997; Van der Niet et al., 2014). Such selection has been shown to operate even within species (Robertson and Wyatt, 1990). At larger geographic scales or along environmental gradients, pollinator-mediated selection is a reasonable hypothesis for morphological polymorphism if pollinators exhibit habitat preferences or have distributional limits. However, at smaller geographic scales, other factors must also be considered to explain the maintenance of phenotypic variation in *Platanthera* species. Characterizing the geographic scale of phenotypic variation within species is important for distinguishing among competing factors in the maintenance of this variation.

Platanthera dilatata (Pursh) Lind. ex L.C. Beck is distributed across the northern U.S. and Canada, reaching as far south as New Mexico and as far north as Alaska. This species has been treated as representing three varieties based on nectar spur length, which are thought to partition pollinators by corresponding proboscis lengths (Sheviak, 2002). However, as noted by Sheviak (2002), "the recognized varieties of *P. dilatata* are evidentially merely endpoints in a very complex variation pattern," leading to unanswered questions as to why polymorphism in this species exists.

In this study, we examined phenotypic and genotypic divergence among populations of *P. dilatata* in Southeast Alaska and across elevational, climatic, and edaphic gradients. In the study area, *P. dilatata* populations do not readily fit into the varieties outlined by Sheviak (2002). Thus, we sampled across an area covering many habitats and flower types to quantify variation in soil characteristics, climatic variables, flower morphological traits, and genetic variation at microsatellite loci. We used these data to address the following questions: (1) Is flower morphological variation structured at smaller geographic scales, (2) Are floral phenotypes genetically divergent, (3) Does gene flow occur across morphologically distinct populations, and (4) How do geographic distance and environmental differences influence population genetic structure? We predicted strong isolation by distance at the regional scale (i.e., encompassing all study populations) because of limitations on gene flow *via* seeds and selection on flowers by pollinators, but at a local scale (i.e., less than 50 km between populations), we predicted that environmental factors would more strongly influence genetic structure because seeds should be capable of dispersal over these distances but may differ in adaptation to habitats and pollinators.

2. Materials and methods

2.1. Study area

This study took place in Southeast Alaska, United States. which comprises an 800 km mountainous coastline and adjacent island chain along the northwest coast of North America (Figure 1). The climate of this region is primarily wet maritime, averaging over 300 cm annual precipitation. The average maximum temperature reaches 18°C in July, and the average minimum temperature reaches -4°C in January (Shulski and Wendler, 2007; Bienek et al., 2012). The predominant coastal vegetation is northern rainforest; about 17% of the area is non-forested shrubland and peatland (Kirchoff et al., 2016). This area was glaciated <10,000 years BP; as a result, climate and post-glacial migration strongly affect vegetation composition (Andersen, 1955; Mathewes, 1985), but glacial refugia present during the late Wisconsin glaciation also may have allowed persistence and recolonization of vegetation within this region (Carrara et al., 2007).

In the study area, *P. dilatata* is most abundant in open bog and fen peatlands, coastal, lakeshore, and riverine meadows, and anthropogenic-disturbed roadsides (Figure 2). Bogs, also known as muskeg, are usually ombrotrophic and develop at low to mid-elevations but grade into subalpine conditions with less organic matter. These habitats usually comprise sapric to hemic peat, and support plant species of open bogs, including *Sphagnum* L. sp., *Carex* L. sp., and Ericaceous shrubs (Neiland, 1971). Fens usually occur at low elevations along drainage ways and range from weak to moderately minerotrophic, receiving greater nutrient input than bogs (Fellman and D'Amore, 2007; Fellman et al., 2008; D'Amore et al., 2010, 2015). They comprise floating or solid mats of hemic to fibric peat and support a subset of bog and meadow vegetation. Coastal meadows, also termed uplift meadows, are developed in fine-textured glacial outwash and lacustrine deposits and are undergoing isostatic uplift following glaciation. They are dominated by broad-leaved herbs, with a minor component of graminoid species, and may zonate along tidelands (Stone, 1993). Anthropogenic roadsides have mineral soils developed from grading and gravel deposition and tend to represent a subset of meadow vegetation that tolerates disturbances such as seasonal mowing.

The mycorrhizal fungi *Ceratobasidium* sp. and *Tulasnella* sp. have been identified in *P. dilatata* root samples from the study area. Two of three *Ceratobasidium* isolates were from muskeg, while 10 of 11 *Tulasnella* samples were from fen, meadow, or anthropogenic habitat (Melton, 2020; M. McCormick, pers. comm.; L. Zettler, pers. comm.).

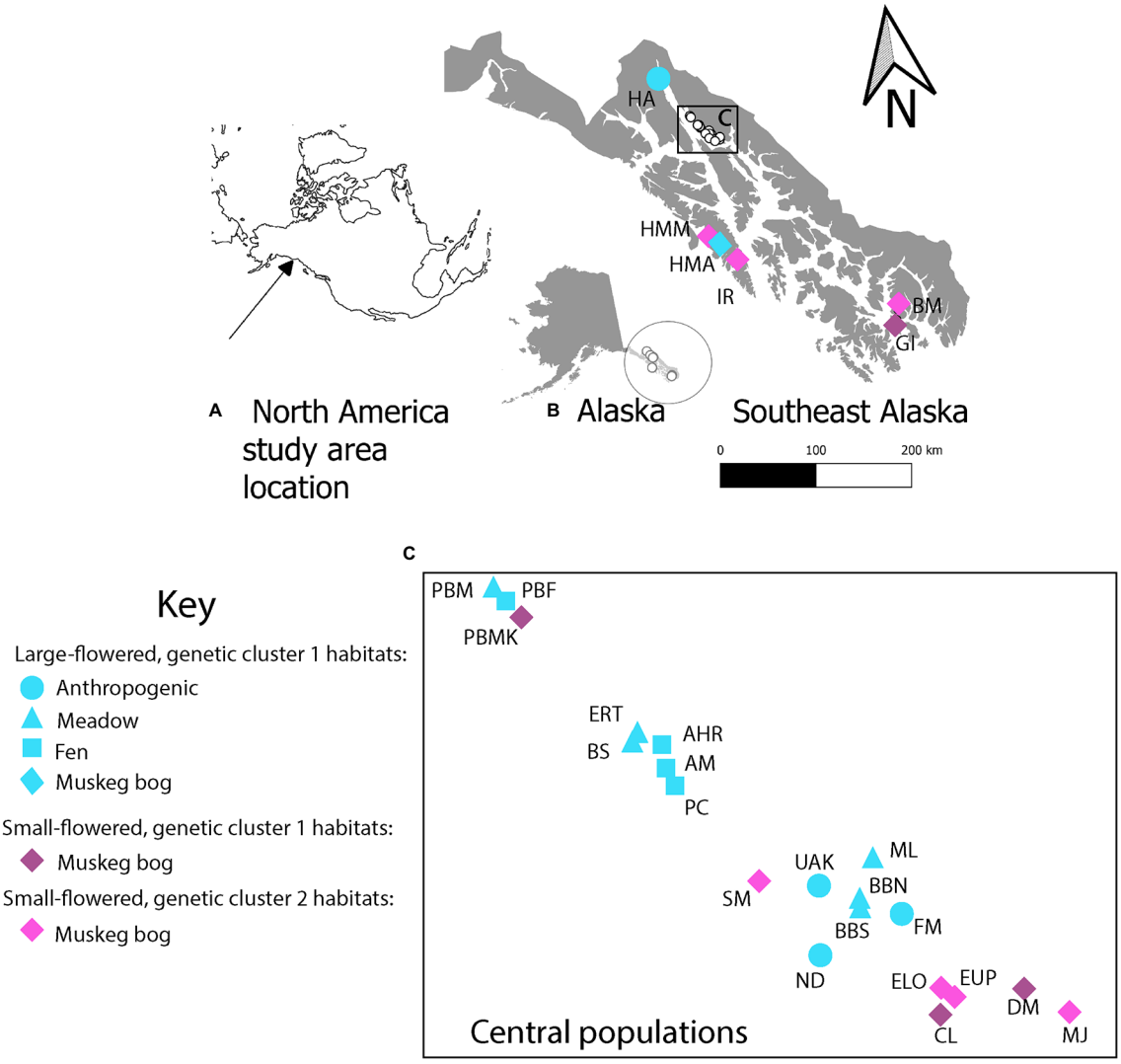

FIGURE 1
Location of **(A)** study area in North America, **(B)** Southeast Alaska collection sites of *Platanthera dilatata* sampled for morphology, genetic markers, and soil characteristics, and **(C)** distribution of populations shown in the box in panel B and referenced as central in landscape genetic analyses. In panels B and C, shapes and colors indicate the six combinations of habitat x flower size x genetic cluster observed in this study. Site names follow those in Table 1 and Supplementary Table S1.

Pollinators of *P. dilatata* include Noctuidae moths, the hawkmoth *Hyles gallii* (Rottemburg, 1775), and the butterfly *Pieris marginalis* Scudder, 1861 (Figure 3); pollinia were deposited on the proboscises of these insects (Bowles and Armstrong, 2021). Noctuidae moths appear to be primary pollinators across all habitats, but hawkmoths may be most frequent in fens and meadows.

2.2. Site selection

The 26 study sites represented 12 muskeg bogs, six meadows, four fens and four anthropogenic roadsides, spanning *ca.* 500 km from north to south (Figure 1; Table 1; Supplementary Table S1). Although fen, meadow, and anthropogenic habitats may occur in southern Southeast Alaska, study sites for these habitats were restricted to northern Southeast Alaska. Sites were selected based on accessibility, lack of anthropogenic disturbance (excluding roadsides) presence of >10 flowering plants at each site, and regional distribution to maximize sampling in morphologically diverse populations and environmentally variable sites.

2.3. Morphological data collection and analysis

Lip and spur length are the most important variables for distinguishing among varieties of *P. dilatata* (Adhikari and Wallace, 2014). These metrics were obtained from single flowers selected from 10–28 (mean = 17.4, se = 1.01) inflorescences from each study population. Flowers were collected in 2018–2019. Flower collection was stratified to represent the range of inflorescence sizes present; flowers were collected from the lower third of inflorescences to avoid nectar spurs that were not fully developed. Flowers were stored in zip-lock plastic bags at 4°C, and measured within 48 h. Each flower

TABLE 1 Genetic diversity at eight microsatellite loci across sampled locations of *Platanthera dilatata* in Southeast Alaska.

Site name	Habitat	N	Na	%P	H_O	H_E	F_{IS}
Large-flowered populations							
FM	Anthropogenic	18.4	3.0	100	0.413*	0.497	0.106
HM	Anthropogenic	20.0	3.4	100	0.500	0.496	0.031
ND	Anthropogenic	23.6	4.5	100	0.516	0.604	0.034
UAK	Anthropogenic	19.0	3.7	100	0.507	0.501	0.033
BS	Meadow	22.0	3.6	88	0.381*	0.458	0.113
BBN	Meadow	15.9	3.6	100	0.557	0.528	0.039
BBS	Meadow	22.7	3.9	100	0.460*	0.513	0.088
ERT	Meadow	15.0	3.6	100	0.467*	0.511	0.068
ML	Meadow	9.0	3.5	88	0.542	0.542	0.054
PBM	Meadow	9.0	3.0	100	0.403*	0.428	0.133
AHR	Fen	12.6	3.6	100	0.544	0.513	0.090
PC	Fen	22.6	3.7	100	0.432*	0.501	0.078
AM	Fen	24.0	3.6	100	0.458	0.462	0.043
PBF	Fen	10.0	2.9	100	0.400	0.376	0.051
HMA	Muskeg bog	10.0	1.5	50	0.288	0.226	0.025
Small-flowered populations							
DM[a]	Muskeg bog	24.0	4.0	100	0.641	0.578	0.013
PBMK[a]	Muskeg bog	20.7	3.5	100	0.459	0.491	0.040
CL[a]	Muskeg bog	23.0	4.2	100	0.565	0.600	0.035
GI[a]	Muskeg bog	16.0	2.7	100	0.539	0.470	0.033
ELO	Muskeg bog	20.9	3.2	100	0.431*	0.508	0.069
EUP	Muskeg bog	19.0	2.5	75	0.270	0.284	0.053
HMM	Muskeg bog	21.7	2.2	88	0.319	0.364	0.032
IR	Muskeg bog	23.9	2.7	100	0.350	0.385	0.031
MJ	Muskeg bog	21.0	2.7	88	0.310	0.316	0.043
BM	Muskeg bog	14.6	3.1	100	0.434	0.436	0.044
SM	Muskeg bog	19.0	2.6	100	0.395	0.451	0.071
Mean–large-flowered populations		16.9	3.4	95	0.458	0.477	0.066
Mean–small-flowered without hybrid populations[a]		20.0	2.7	93	0.358	0.392	0.049
T-test[b] *P*		--	2.486 0.022	0.369 0.72	3.074 0.006	2.206 0.039	1.228 0.233

N = mean number of individuals sampled across all loci, Na = mean number of alleles per locus, % *P* = percentage of polymorphic loci, H_O = observed heterozygosity, H_E = expected heterozygosity, F_{IS} = inbreeding coefficient.
*Significant deviation from Hardy–Weinberg equilibrium ($P < 0.05$).
[a]Small-flowered populations suspected of having introgression from large-flowered populations.
[b]*T*-tests were conducted without the inclusion of hybrid small populations.

was dissected to remove the lip and spur, and their lengths were measured to the nearest 0.5 mm. Most spurs were falcate, and they were flattened under a flexible sheet of transparent plastic for linear measurement.

Population means (+ se) were calculated for flower and lip length. To assess whether morphological groups could be identified, a k-means cluster analysis was performed in NCSS statistical software (Hintze, 2013). This test evaluated 1–5 clusters, using 5 random starts to produce an optimum solution in which within-cluster sum of squares is minimized. A goodness of fit comparison of the percent variation in each within-cluster group sum of squares relative to one group was used to evaluate which number of clusters had the greatest reduction in variation (Hintze, 2013). The Duda and Hart (1973) test was also used to evaluate whether single or multiple clusters better fit the data, followed by application of the Calinski and Harabasz (1974) index to further evaluate the most likely number of clusters beyond one.

We calculated P_{ST} (Brommer, 2011) to estimate the degree of differentiation in lip and spur length among populations. P_{ST} was then compared to F_{ST} estimated from the microsatellite data (see below) to evaluate the relative potential for selection and genetic drift to drive the observed differences in floral traits. P_{ST} was estimated separately for lip length and spur length using the R package Pstat (Blondaeu Da Silva and Da Silva, 2018). Data were subjected to Atchinson transformation and the value of c/h^2 was set to 1; bootstrap analysis with 1,000 replicates was used to calculate 95% confidence intervals for P_{ST} and this was compared to our estimate of F_{ST} based on microsatellite loci.

2.4. Soil data collection and analysis

Soil samples were collected from each study site in 2018–2022. Each sample comprised multiple excavations made to rooting depth with a hand trowel, which were combined into a single collection for each site. Samples were analyzed by Waypoint Analytical (Richmond, Virginia, USA) for percent organic matter (POM); parts per million (PPM) Ca, K, Mg, and P; percent base saturation (PBS) Ca, K and Mg; percent H saturation (PHS); and cation exchange capacity (CEC, meq/100 g). Analytic methods followed Horton (2011).

Soils data were analyzed with ANOVA and multivariate statistics. One-way ANOVA was used to test whether soils variables differed among muskeg, fen, meadow, and anthropogenic habitat groups, which supported different orchid phenotypes (see below). For these tests, transformations were used to approximate normality for POM and PBS K (arcsin transformation), PPM P (log transformation), PPM Ca and Ca (square root transformation). Non-metric Multidimensional Scaling (NMS) was used on PCORD (McCune and Mefford, 2011) to ordinate habitat groups using POM, pH, CEC, PPM P, PBS K, PBS Mg, PBS Ca, and PHS as metrics. A relative Euclidian distance measure with a random seed starting configuration and 100 runs with real data were used to project three axes using a Varimax rotation, for which stability was tested with a randomization test. Relationships of each metric with the first and second NMS axis were tested with correlation analysis. A Multi-Response Permutation Procedures (MRPP) test was used on PCORD to assess whether habitats differed in their multivariate distributions based on soils metrics. Because of skewed metrics in anthropogenic habitat soils, the MRPP test was repeated with this group excluded from the analysis.

2.5. Genetic data collection and analysis

Leaf samples used in genetic analyzes were collected in 2018–2019. A 5 cm length of fresh leaf tissue was removed from one leaf

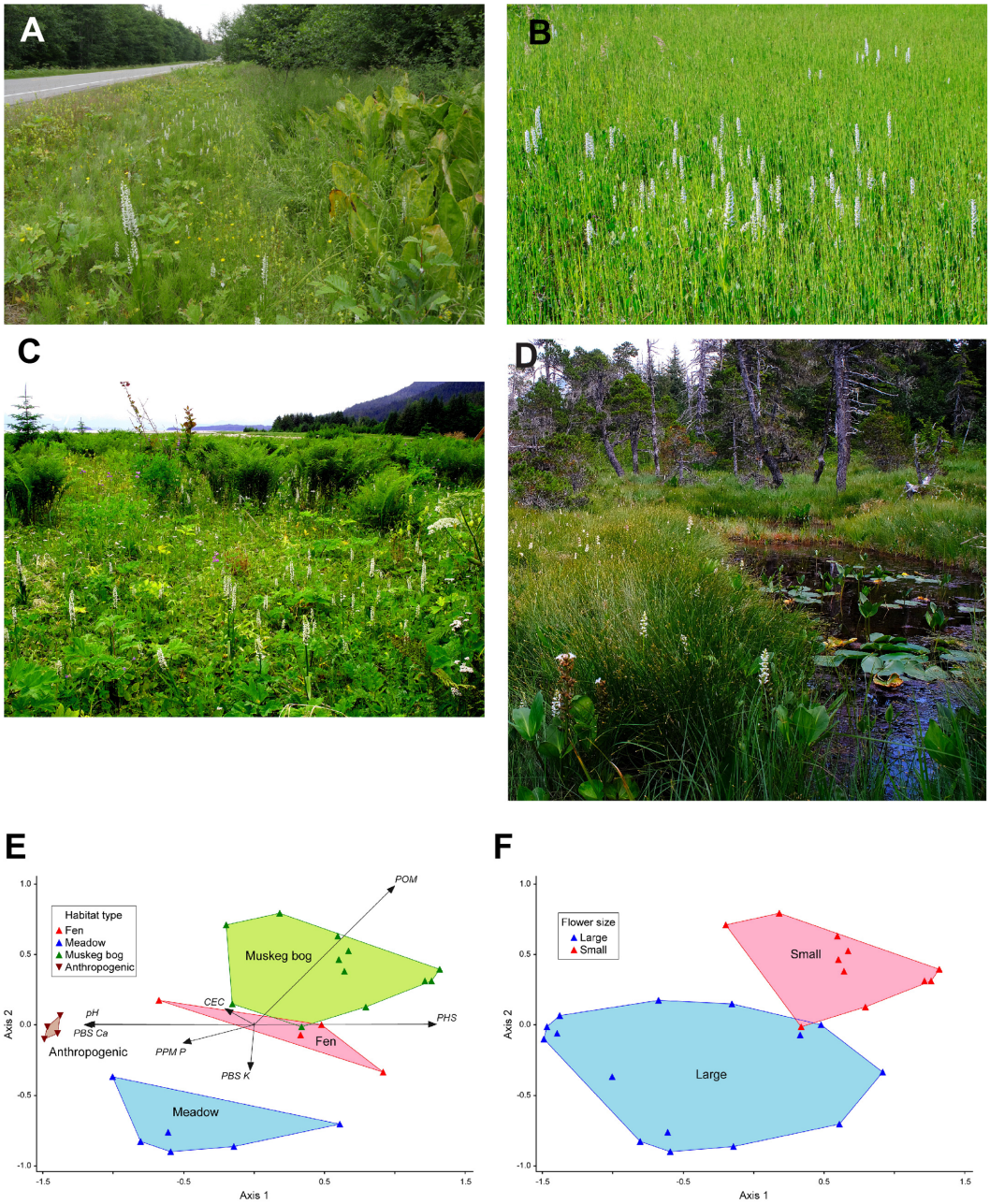

FIGURE 2
Variation in habitats and soils occupied by *Platanthera dilatata*. **(A)** anthropogenic roadside, **(B)** fen, **(C)** uplift meadow, **(D)** and muskeg bog. Panels **(E,F)** show the NMS ordination of habitat vegetation types in relation to soil characteristics and flower group as indicated by the K-means clustering analysis of floral traits **(F)**. Ordination final stress=3.4891, final instability=0.0; probability of final stress obtained by chance (Axis 1 p=0.002, Axis 2 p=0.044). Cumulative correlations between ordination distances and distances in the original n-dimensional space: Axis 1 r^2=0.828, Axis 2 r^2=0.990. MRPP: all habitats (A=0.41613688, p<0.0001); anthropogenic habitats excluded (A=0.26071168, p<0.0001). See Supplemental Table S4 for soils variables axis correlation statistics.

from 9–26 (mean = 18.8 se = 1.3) plants from each study site. Leaf samples were stored in zip-lock plastic bags at 4°C. These samples were dried within 24 h. by placing them in folded aluminum foil containing silica gel crystals and then sealed within double zip-lock plastic bags. DNA was extracted from dried leaves using the SYNERGY 2.0 Plant DNA extraction kit (OPS Diagnostics, Lebanon, New Jersey, USA) and stored in 1X TE buffer. DNA samples were standardized to 10 ng/μl for use in PCR. Each sampled plant was genotyped at nine microsatellite loci that were developed from a transcriptome library of *P. dilatata* (Wallace, unpublished data). Primer sequences are provided in Supplementary Table S2. The nine loci were amplified using a multiplex PCR with the Kapa 2G Fast multiplex PCR kit (Roche Sequencing and Life Science, Wilmington, Massachusetts, USA) and fluorescent labeled primers following protocols in Culley et al. (2013). Each fluorescently labeled primer contained a sequence that matched a tag sequence located on the 5′

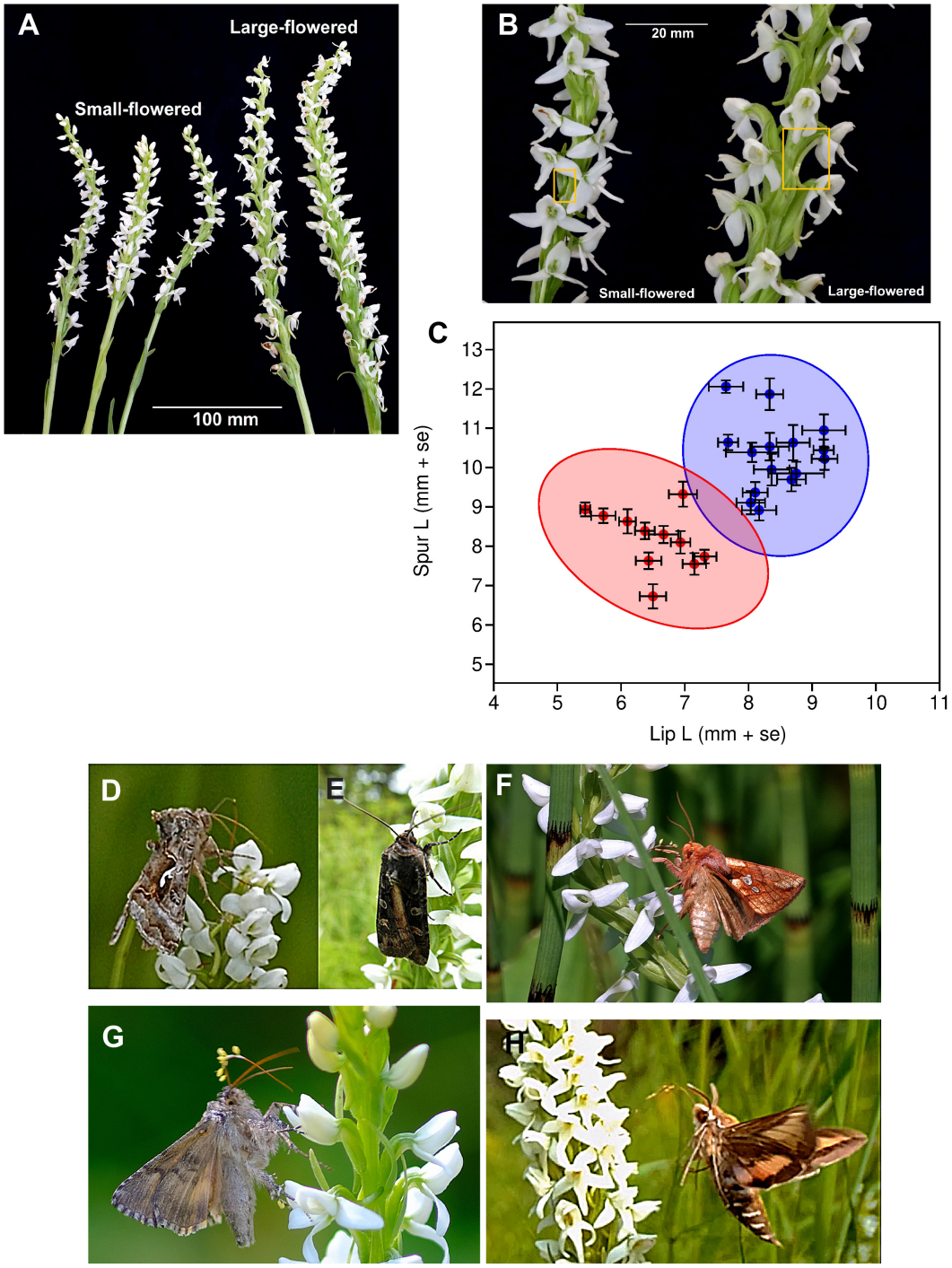

FIGURE 3
Variation in flower traits and pollinators of *Platanthera dilatata* in the study area of Southeast Alaska. Differences in **(A)** inflorescence size and **(B)** the lengths of spurs (in boxes) between plants grouped by flower size and **(C)** mean (+ se) lip and spur length of flowers, with the ellipses indicating 95% concentrations of populations within large and small-flowered groups identified by k-means cluster analysis. Pollinators observed on *P. dilatata* flowers in the study populations include **(D)** *Autographa corusca* Strecker, 1885 on small-flowered phenotype (photo: R. H. Armstrong), **(E)** *Actebia fennica* (Tauscher, 1806) on small-flowered phenotype (photo: G. Bayluss), **(F)** *Plusia* sp. Ochsenheimer 1816 on the large-flowered phenotype (photo: R. H. Armstrong), **(G)** *Autographa corusca* Strecker, 1885 on large-flowered phenotype, and **(H)**, *Hyles gallii* (Rottemburg, 1775) on large-flowered phenotype (photo: R. H. Armstrong); D-G are Noctuidae species, and H is a Sphingidae species. Large-flowered group: lip mean=8.43 (se=0.13), spur mean=10.31 (se=0.23), *t*-test of lip and spur lengths: $t=-7.089$, $p<0,001$; small-flowered group: lip mean=6.49 (se=0.173), spur mean=8.11 (se=0.25),), *t*-test of lip and spur lengths: $t=-5.385$, $p<0.001$. One-way ANOVA between morphological groups: Lip $F_{1,24}=85.45$, $p<0.0001$, Spur $F_{1,24}=40.50$, $p<0.0001$. Lip-spur correlations: among groups ($r=0.6409$, $p=0.0004$); small-flowered group ($r=-0.3877$, $p=0.237$); large-flowered group ($r=-0.0581$, $p=0.8435$).

end of the locus-specific forward primer. For each sample, each multiplex reaction was performed in a final volume of 10 µl in the presence of 10 ng of template DNA, 100 µmole of each of the reverse and tagged fluorescently labeled primers and 10 µmole of tagged forward primer using KAPA 2G Fast Multiplex PCR mix. The thermal cycler program used to amplify loci included 3 min at 95°C, 30 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C, and a final extension step of 1 min at 72°C. Amplified products were genotyped at the Institute of Biotechnology at Cornell University with LIZ 500 size standard, and individual alleles were sized using GeneMarker (SoftGenetics, State College, Pennsylvania, United States).

The presence of null alleles in each locus and population was checked using the program FreeNA (Chapuis and Estoup, 2007). Null allele frequencies <0.2 are not expected to greatly influence the results of population genetic analyzes (Dakin and Avise, 2004; Carlsson, 2008). Thus, we considered further only loci exhibiting a null allele frequency > 0.2, which occurred at three loci, 72267, 99945, and 107223, in eight, three, and three populations, respectively. Locus 72267 was removed from the dataset because of the extensive occurrence of potential null alleles. We further investigated inbreeding as a potential cause of null alleles for the other two loci. Heterozygote deficiency, which is a potential sign of null allele presence, has often been reported in association with significant F_{IS} in other orchids (Chung et al., 2004; Alcantara et al., 2006; Andriamihaja et al., 2021). For each of the five populations suspected of having null alleles, we compared a model based on the inclusion of null alleles, inbreeding, and genotyping errors (i.e., nfb) with one lacking inbreeding (i.e., nb) using the software INEST v. 2.2 (Chybicki and Burczyk, 2009). These analyzes were implemented using a Bayesian approach with 1 million MCMC cycles, keeping every 100th result, and a burn-in of 10,000 prior to summarizing the results. DIC values were compared between the two models to evaluate the impact of inbreeding on observed diversity. For population PC, the full model had a substantially lower DIC than the model without inbreeding. For the other four populations (i.e., HMM, IR, SM, and ND), the difference in DIC between the two models was less than 1.5. As these results suggest that inbreeding may account for the lack of heterozygous individuals in these populations at the suspected loci, we chose to retain data for these locus-population combinations for further analysis of genetic diversity and structure.

Within each population, we tested for significant departures from Hardy–Weinberg expectations using a global test of heterozygote deficiency in GENEPOP version 3.2 (Raymond and Rousset, 1995; Rousset, 2008). Genotypic linkage disequilibrium was measured for each pair of loci in each population and tested through Fisher's exact test using GENEPOP version 3.2 (Raymond and Rousset, 1995; Rousset, 2008) and applying a Bonferroni correction (Holm, 1979). Genetic diversity within populations was assessed as number of alleles per locus (N_a), observed heterozygosity (H_O), expected heterozygosity (H_E), and percent of polymorphic loci (% P) using GenAlEx version 6.503 (Peakall and Smouse, 2012). Inbreeding coefficients were calculated in INEST (Chybicki and Burczyk, 2009) as described above. To determine if genetic diversity varies between large and small-flowered populations, as identified in the morphological K-means clustering, we compared mean values of N_a, H_O, H_E, and F_{IS} using *t*-tests in SPSS v. 27 (IBM Corp, 2020). $p < 0.05$ was used to identify significant differences in genetic diversity between the flower groups.

We evaluated population genetic structure according to the groups identified by morphological analyzes and NMS of the soil variables, that is, between large-flowered and small-flowered groups and separately among the four habitat types (Table 1) using analysis of molecular variance (AMOVA) (Excoffier et al., 1992), conducted in GenAlEx version 6.503 (Peakall and Smouse, 2012). Statistical significance of AMOVA was assessed by 9,999 permutations. We used the Bayesian clustering approach implemented in STRUCTURE v. 2.3.4 (Pritchard et al., 2000) to test for admixture and assignment of individuals to distinct genetic clusters. These analyzes were conducted using an admixture model with correlated allele frequencies, a 'burn-in' period of 50,000 MCMC replicates, sampling 100,000 replicates, and eight iterations of each K value, from one to 13. This range of K values was used in the final run because an initial analysis of four iterations each for K values from 1 to 25 under similar run parameters indicated low probability of a K value greater than five. For the final analysis multiple posterior probability values (log likelihood (lnL) values) for the eight iterations of each K were generated, and the most likely number of clusters was determined using STRUCTURE HARVESTER (Earl and vonHoldt, 2012) and Delta K- (Evanno et al., 2005). CLUMPP (Jakobsson and Rosenberg, 2007) was used to aggregate individual assignment probabilities from the eight iterations for the selected K. STRUCTURE PLOT (Ramasamy et al., 2014) was used to generate plots of individual assignment from the CLUMPP output file.

We estimated the potential for admixture in populations using several methods. Identify scores (Q-matrix scores) from the STRUCTURE analysis were used to infer if individuals were of pure ancestry or contained an admixed background. An identity score < 0.9 in a single cluster was used to assign an individual as admixed. NewHybrids v1.1 (Anderson and Thompson, 2002) was used to assign each individual to one of six genotypic classes (i.e., pure large-flowered, pure small-flowered, F1, F2, backcross with large-flowered, or backcross with small- flowered). This analysis was conducted without specifying individuals to a particular class, and all individuals were analyzed. We ran the analysis using a Jeffreys prior, 10,000 burn-in replicates, and 1 million sweeps before assignment probabilities were determined. No individual was assigned to any of the hybrid classes with probably >0.7, so we only considered a hybrid group, rather than F1, F2, or backcross generations. Furthermore, we used a cut-off probability of >0.9 to assign individuals into one of the pure parental groups, rather than the hybrid group.

BayesAss (Wilson and Rannala, 2003) and Geneclass 2 (Piry et al., 2004) were used to estimate the proportion of immigrants and non-immigrants. Whereas BayesAss (Wilson and Rannala, 2003) is better able to detect older instances of movement, Geneclass (Piry et al., 2004) more aptly identifies first generation immigrants. For these analyzes we assigned populations to one of three groups, large-flowered populations, small-flowered populations, and hybrid populations, after considering the morphological groupings and genetic assignments suggested by STRUCTURE and NewHybrids (see results, Supplementary Table S4; Figures 3C, 4A). Hybrid populations were identified by their conflicting placement into groups based on morphological and genetic variation (i.e., small-flowered plants that were genetically similar to large-flowered plants). BayesAss analysis was conducted using 50 million iterations, a burn-in of 1 million, and sampling every 5,000 generations. The Geneclass analysis was conducted to identify first generation

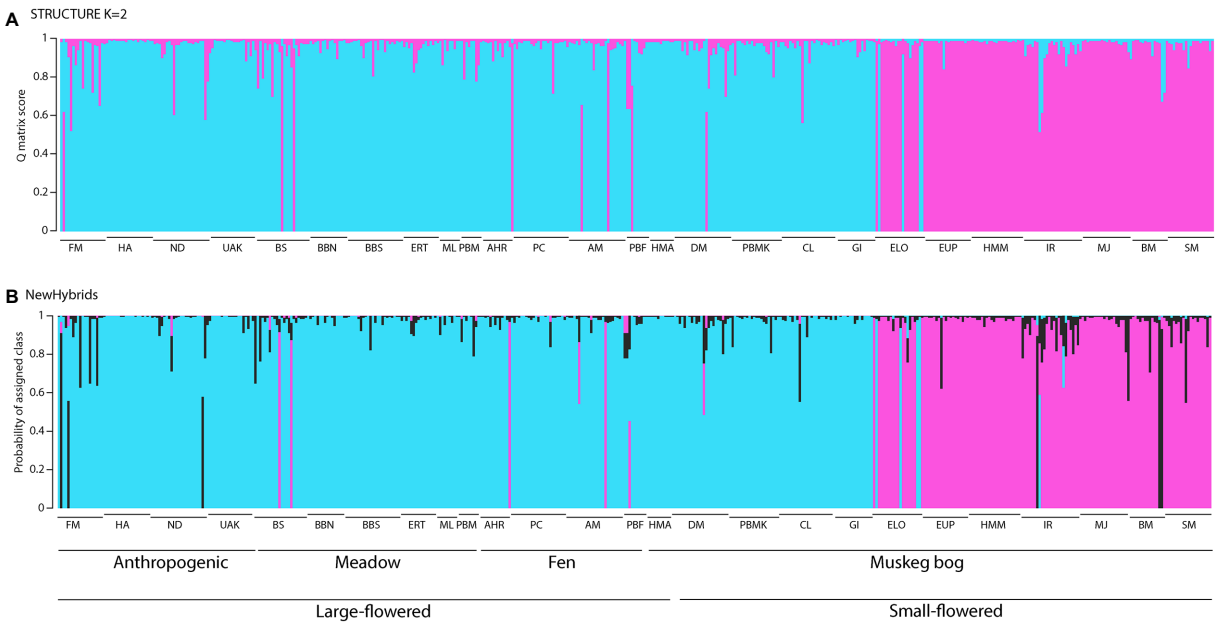

FIGURE 4
Admixture proportions for all samples of *Platanthera dilatata* from Southeast Alaska based on analysis with **(A)** STRUCTURE and **(B)** NewHybrids. For a, the Q-matrix scores for each individual according to the solution $K=2$ (blue and pink clusters) are indicated. For **(B)**, the estimated probability that an individual is from one of the pure parental groups (blue or pink) or a hybrid (black) is shown. Populations are arranged in the order shown in Table 1. See Figure 2E for alignment of habitat groups along soil gradients.

immigrants only using the criterion of Rannala and Mountain (1997) with 10,000 simulated individuals under the simulation algorithm of Paetkau et al. (2004). A $p<0.05$ was used to identify significant immigration events.

Past studies (e.g., Balkenhol et al., 2009; Kierepka and Latch, 2015) have found that different methods for assessing the role of geography, environment, or other factors on population genetic structure show only moderate agreement and recommend choosing multiple statistical approaches when testing for isolation by distance (IBD), isolation by environment (IBE), or other factors. Thus, we used multiple matrix regression (MMR) (Wang, 2013) and distance-based RDA (dbRDA) (Legendre and Anderson, 1999) to test for IBD and IBE. MMR compares pairwise population genetic distance against distance matrices based on explanatory variables using regression, whereas dbRDA assesses selected explanatory variables directly as predictors of population genetic distance. We conducted these analyzes at a regional scale, i.e., on all populations, as well as a local scale, i.e., the centrally located populations near Juneau (Figure 1B) to evaluate whether environmental factors are more predictive of genetic structure at the local scale and geographic distance at the regional scale, given that orchid seeds may be capable of dispersing over several hundred kilometers (Arditti and Ghani, 2000; Phillips et al., 2012).

For all analyzes, pairwise population genetic distances were generated using the distance metric of Cavalli-Sforza and Edwards (1967), with correction by the INA method implemented in FreeNA and described in Chapuis and Estoup (2007). For MMR, distance matrixes reflecting geography and environmental features were created in the following manner. The pairwise population geographic distance matrix was created using GPS coordinates and the distGeo() function in the geosphere package v. 1.5 (Hijmans et al., 2021) in R (R Core Team, 2022). These geographic distances were log-transformed to reduce the impact of the largest distances. For the environmental dataset we considered elevation, four soil factors that were important in the NMS ordination (i.e., POM, pH, Pppm, and PBSK), and five uncorrelated ($r^2<0.70$) climate variables (i.e., precipitation in the warmest quarter, precipitation seasonality, mean temperature of the coldest quarter, mean temperature of the driest quarter, and mean temperature of the wettest quarter) sampled from 30-s layers of the WorldClim data set (Fick and Hijmans, 2017) for all locations considered in this study. The environmental dataset was subjected to principal component analysis (PCA) using R (R Core Team, 2022). Values for each site along the first two axes, which accounted for 49% of the observed variation, were used to construct an environmental distance matrix based on Euclidean distances in Passage 2 (Rosenberg and Anderson, 2011). Multivariate MMR was conducted using the MMRR script of Wang (2013) in R (R Core Team, 2022) using the explanatory matrixes of geographic distance and environmental distance and the response matrix of genetic distances. Significance was tested using 9,999 permutations.

For dbRDA, the geographic distance matrix was used in a principal coordinates of neighbor matrices (PCNM; Borcard and Legendre, 2002; Borcard et al., 2004) in R (R Core Team, 2022) with default threshold values to generate a set of independent variables reflecting spatial relationships among the populations. The positive PCNM axes were retained and tested as predictors of genetic distance in dbRDA. To test for IBE, we used the first two axes from a principal component analysis (PCA) of the environmental variables, as described above, as predictors of pairwise genetic distances. The explanatory variables were assessed independently in marginal tests and conditioned on geographic distance to account for potential correlation between environmental factors and geography. The

dbRDA analyzes were conducted using the capscale () function of the Vegan package (Oksanen et al., 2020) in R (R Core Team, 2022). An analysis of variance was used to evaluate significance of each model. The varpart() function in R (R Core Team, 2022) was used to assess the contribution of each environmental and geographic variables to genetic distances.

3. Results

3.1. Flower morphology

In the k-means cluster analysis, a two-group solution reduced percent variation of within-sum of squares to 29.6%. Subsequent clusters further reduced variation by <10%. The Duda-Hart test indicated that the optimal clustering solution contained more than one group ($DH_K = 0.3555$, alpha = 0.99), and the Calinski-Harabasz index also was greater for two clusters. Thus, two clusters were selected as the optimal solution.

The two-cluster analysis separated populations with significantly different lip and spur lengths, which were correlated among, but not within, groups (Figure 3C). In both groups, spurs were significantly longer (by >20%) than lips, however, one group had 20% longer lips and spurs than did the other group, and thus larger flowers (Figures 3A,B). A comparison of inflorescence size among three populations of small-flowered plants and four populations of large-flowered plants (among three habitats) found that small-flowered plants also had smaller inflorescences ($N = 37$, mean = 26.95, se = 1.60) than did large-flowered populations ($N = 52$, mean = 38.85, se = 2.25); nested ANOVA of ln-transformed data: $F_{2,82} = 28.77$, $p = 0.033$. The correspondence between habitats and flower morphology is shown in Figure 2F. The group with smaller flowers comprised populations occurring only in muskeg bog habitat (Figure 2D). The group with larger flowers included all fen, meadow, and anthropogenic habitats, as well as a single muskeg site that occurred at high elevation and is a transition to alpine habitat (Figures 2A–D).

Populations showed strong differentiation in spur length and lip length as P_{ST} values were 0.94 (95% CI: 0.930–0.959) for spur length and 0.97 (95% CI: 0.969–0.980) for lip length. These estimates were robust to variation in our selection of the value for c/h^2. The critical value of P_{ST}, whereby quantitative traits are more strongly reflective of selection than genetic drift, occurred at $c/h^2 < 0.5$ (Supplementary Figure S1).

3.2. Soil chemistry and fertility

Most soils variables differed significantly among habitat groups (Supplementary Table S3). Percent organic matter had the strongest differentiation. It was significantly greater in muskeg (64%) intermediate in fen (37%), and lower (< 10%) in meadow and anthropogenic habitats. Other significant variables (pH, CEC, ppm Ca, and PBS Ca) were greater in anthropogenic habitat and not different among other habitats. Ca was about 400% higher in anthropogenic habitat, where it reached 1783.5 PPM. Though it did not differ significantly ($p = 0.115$), PBS K tended to be higher in meadow habitat, where it reached 4.9%.

Non-metric Multidimensional Scaling reached a stable solution for ordination of two axes after 88 iterations (Figure 2E). Only CEC and PBS Mg were not significantly correlated with either ordination axis (Supplementary Table S4). On Axis 1, muskeg bog habitat was strongly associated with positive axis scores and highly correlated with increasing POM and PHS, while anthropogenic roadside habitat was strongly associated with negative axis scores and highly correlated with increasing pH and PBS *Ca*. Meadow habitat was most strongly associated with Axis 2 and greater PBS K, but also tended to be associated with increasing PPM P along Axis 1. Fen habitat was centrally located and intermediate with respect to soil chemistry and nutrients. With MRPP, all habitats had significantly different multivariate distributions, which remained different with anthropogenic habitat excluded from the model (Figure 2E).

3.3. Genetic variation and population structure

Among the 728 inter-locus comparisons, there were six instances of significant genetic disequilibrium identified in three populations. No locus pairs exhibited significant disequilibrium in multiple populations, but one population (i.e., ELO) did have four loci out of equilibrium. Overall, these results suggest that the loci are genetically independent, and that instances of linkage disequilibrium are likely due to demographic factors unique to the affected populations. Seven populations had a deficiency of heterozygotes consistent with deviation from Hardy–Weinberg equilibrium (Table 1). Mean genetic diversity was significantly higher in populations assigned to the large-flowered group than those assigned to the small-flowered group (excluding the four hybrid populations) when considering N_a, H_O, and H_E but not for % P or the inbreeding coefficient (Table 1).

AMOVA assigned most of the observed genetic structure within populations (79–80%), then among populations (15–16%), and between the small and large-flowered groups (6%) or among the habitats (5%; Table 2). All F-statistics were significant ($p < 0.01$). The optimal number of groups, based on Bayesian analysis in STRUCTURE of the genetic variation, was two clusters (Figure 4A). The two clusters primarily align with large and small-flowered populations, although samples from four small-flowered populations (i.e., PBMK, CL, BM, and DM) were placed in the cluster with large-flowered populations. Most individuals (80%) were assigned to a single cluster with Q-matrix values >0.95.

Our analyzes indicated admixture between the phenotypic groups. This was most extensive for the four small-flowered populations that are genetically-like large-flowered populations as all but seven samples from these populations were assigned to this cluster with Q-matrix scores >0.9 by STRUCTURE, but other instances of admixture were also noted for most populations (Figure 4A). In fact, based on a cut-off of 0.9 in Q-matrix scores from STRUCTURE, all but four populations (i.e., HA, HMA, HMM, and ML) contained at least one admixed individual, resulting in *ca.* 10% of all individuals assigned as admixed. NewHybrids produced nearly identical results to those from STRUCTURE (Figure 4B), although seven populations were not predicted to contain admixed individuals by this analysis. NewHybrids also estimated more extensive hybridization in the small-flowered IR population compared to

TABLE 2 Results from an analysis of molecular variance based on allelic diversity among populations of *Platanthera dilatata* from Southeast Alaska.

Source	df	SS	MS	Percent of variance
Flower groups				
Among groups	1	92.542	92.542	6
Among populations	24	383.237	15.968	15
Within populations	936	1830.080	1.955	79
Total	961	2305.860	2.494	100
$F_{RT} = 0.063$, $P < 0.001$; $F_{SR} = 0.163$, $P < 0.001$; $F_{ST} = 0.216$, $p < 0.001$				
Habitat				
Among groups	3	122.659	40.886	5
Among populations	22	353.120	16.051	16
Within populations	936	1830.080	1.955	80
Total	961	2305.860		100
$F_{RT} = 0.045$, $P < 0.01$; $F_{SR} = 0.165$, $p < 0.01$; $F_{ST} = 0.202$, $p < 0.01$				

Groups were designated by (a) flower size and (b) habitat.

STRUCTURE, which identified admixed genotypes in only four individuals in this population.

Further support of two genetically divergent groups was found in the estimated immigration rates, which were extremely low between large-flowered and small-flowered populations. Both BayesAss and Geneclass suggested that at least 97% of the individuals in each of these groups originated within their assigned group (Supplementary Table S5). BayesAss, but not Geneclass, indicated strongly unidirectional immigration from the large-flowered group into the hybrid populations. Only a low level of immigration was detected into the hybrid group from the small-flowered group, even though these populations share a common habitat type and are morphologically similar. Additionally, low levels of recent immigration were detected between large-flowered and small-flowered groups and from the hybrid group by GeneClass.

Landscape genetic analyzes indicated that at the regional level, both geographic distance and environmental factors are predictive of genetic structure. In MMR analysis at this scale, the multivariate model placed geographic distance as the strongest factor, with environmental distance slightly less important but still significant. The overall r-square for this model is 0.27, but it is significant ($p = 0.0002$). Comparable results were obtained with dbRDA, with geographic distance explaining slightly more variation than environmental factors when considered independently (Table 3). Despite a correlation between geographic distance and environmental distance, environmental factors do remain significant in the dbRDA conditioned on geographic distance. At a smaller local scale, geographic distance was not a significant factor explaining genetic structure among populations, but environmental factors were in both MMRR and dbRDA (Table 3; Supplementary Figure S3).

TABLE 3 Results from distance-based Redundancy Analyzes (dbRDA) testing the effects of geographic distance (Geo) and environmental factors (Env) on genetic distance among the populations of *Platanthera dilatata* surveyed in Southeast Alaska.

	Marginal test			Conditional test		
Variable	*F*	*P*	% Variation	*F*	*P*	% Variation
Full						
Geo	1.904	0.01	26.62			
Env[a]	2.83	0.001	19.75	2.13	0.017	18.30
Central						
Geo	1.341	0.141	20.09			
Env[a]	3.126	0.003	26.89	2.334	0.026	25.00

[a]For conditional tests, the contribution of environmental factors was considered after removing the covariate effects of geographic distance. The analyzes were run on the full set of populations (Full) and the centrally located populations only (Central).

4. Discussion

No previous assessment of *P. dilatata* has concurrently examined genetic structure, morphological diversity, and habitat characteristics in populations with a shared regional geography. Examination of populations at this scale provided greater understanding of intraspecific variation for this species and of the environmental factors that may influence morphological and genetic variation. We note several novel results: (1) flower phenotypes are strongly associated with habitats, (2) there is a deep genetic divergence between small-flowered and large-flowered forms, (3) nevertheless, admixture has occurred between populations harboring different phenotypes and introgression is deeply rooted in some populations, and (4) whereas IBD and IBE both contribute to significant population genetic structure at regional scales, among closely spaced populations, environmental factors are stronger determinants of genetic structure.

4.1. Phenotypic variation in relation to environmental factors

Platanthera dilatata has long been recognized as a morphologically variable species (Luer, 1975; Sheviak, 2002). Within Southeast Alaska, *P. dilatata* populations have variable flower morphology, yet the phenotypes are partitioned by habitat (Figures 1, 2). Plants with inflorescences containing fewer flowers and flowers with shorter lips and spurs have a narrow habitat niche as they are restricted to muskeg bogs, whereas plants with larger inflorescences and flowers with longer lips and spurs have a broader habitat niche, occurring across a habitat gradient that is exclusive of muskeg bogs except at extremely high elevations.

The high estimates of P_{ST} for lip and spur lengths relative to F_{ST} is suggestive of divergent selection on flower morphology. When morphological variation is partitionable across populations, selection by pollinators has been documented as an underlying mechanism promoting its retention in *Platanthera bifolia* (Boberg et al., 2014), *Disa draconis* Sw. (Johnson and Steiner, 1997), and *Gymnadenia odoratissima* (L.) Rich. (Sun et al., 2014). Though

flowers of *P. dilatata* fit the primitive "settling moth" syndrome characteristic of Noctuidae moths (Hapeman and Inoue, 1997), regional differences in primary and secondary pollinators readily occur. This variation includes seven Noctuidae species and a Hesperiidae butterfly in Newfoundland, Canada (Boland, 1993), a Noctuidae species in Oregon, United States (Larson, 1992), three *Bombus* Latreille bumblebee species, a Noctuidae, and a Nymphalidae butterfly in British Columbia, Canada (Van der Voort et al., 2022), and three Noctuidae, a Sphingidae moth, and a butterfly in Southeast Alaska (this study; Bowles and Armstrong, 2021). Such variation in primary and secondary pollinator types and abundance could drive selection for variable flower morphology at regional and local scales.

Nectar spur length in *Platanthera* determines whether an insect can access nectar and how pollinia are attached and pollen are deposited on the stigma, and selection should shift spur length toward pollinators that maximize fitness (Boberg et al., 2014). Such selection could be rapid if pollinators remain constant and gene flow from other populations is infrequent. In this study, Noctuidae moths were the most commonly observed pollinators on both the small- and large-flowered forms at all elevations. The hawkmoth *Hyles gallii* (Sphingidae) was observed only on large-flowered plants at low elevations and appeared to carry greater pollen loads than did Noctuidae pollinators. Because hawkmoths have a longer proboscis (*ca.* 25 mm; Miller, 1997) than Noctuidae moths (< 11 mm; Zenker et al., 2011; Zhang et al., 2021), they may be more effective pollinators for longer-spurred orchids than are Noctuidae (Tao et al., 2018). Based on iNaturalist observations ($n = 27$), the median elevation at which *H. gallii* has been observed in SE Alaska is less than 50 m (range 1–285 m), much lower than the median elevation (215 m, range 10–600 m) of muskegs in the study area. If *H. gallii* is prevalent in non-muskeg habits, then selection for longer spurs is expected to drive flower morphology to match the most efficient pollinator (Johnson and Steiner, 1997; Boberg et al., 2014; Sun et al., 2014) despite counter-selection from the more frequent Noctuidae moth pollinators across the study area. By contrast, if plants in muskeg bogs are visited only by shorter tongued Noctuidae species, then selection is expected to drive flowers toward shorter spurs and lead to adaptation of those plants to muskeg habitat. Absence or rarity of Sphingidae moths at higher elevations could limit gene flow across an altitudinal gradient. Further work is needed to characterize pollinators and their selection for flower size and nectar spur length for *P. dilatata* across its distribution to test this hypothesis of localized selection.

A strong correspondence between phenotype and soil conditions has not been previously noted for this species and suggests the possibility that other factors might also influence phenotypic variation. While phenotypic plasticity often underlies phenotypic variation that aligns with environmental differences (Schlichting, 1986), it has rarely been documented for flower traits (Sultan, 2000; Pélabon et al., 2011), and the alpine population HMA also occurs in a muskeg-like habitat at high elevation yet retains a large-flowered phenotype similar to populations at lower elevations. This suggests that flower size is not a plastic trait, and that soil fertility does not influence flower size. An alternative hypothesis is that the small-flowered phenotype is a stress tolerant poor competitor that is adapted to the skewed soil chemistry of muskeg habitat. Although bog habitats may appear to have adequate base concentrations, most nutrients are bound in OM in peat soils and are not available for plant uptake, especially under acidic conditions (Verhoeven, 1986; Vitt and Chee, 1990). Indeed PHS, which was relatively high in muskeg (Figure 2E), was negatively correlated with CEC ($r = -0.4046$, $p = 0.0403$). Mycorrhizal fungi may increase efficiency of mineral uptake in peat soils and could provide a competitive advantage as well as a favorable germination site for orchids in these habitats (Rasmussen, 1995). Unlike *Ceratobasidium*, which can utilize N from both ammonium and nitrate, *Tulasnella* requires ammonium as a N source (Fochi et al., 2017). Ammonium is the predominant form of N in dissolved nutrient concentrations in bogs and fens our study area, but it is much more highly concentrated in fens (Fellman et al., 2008). This could explain the greater presence of *Tulasnella* in fens in our study area and might suggest that the larger phenotype uses these fungi. However, it is unknown whether obligate relationships exist between the large and small *P. dilatata* phenotypes and different fungal species. Other habitats associated with the large-flowered phenotype also tended to have greater fertility and association with the *Tulasnella* fungus. If these mycorrhizal fungi occur in different environments because of nutrient availability (Fochi et al., 2017; Thixton et al., 2020) and have strong relationships with *P. dilatata* phenotypes, then they could reinforce their habitat selection. Given the importance of mycorrhizae to orchid life history, research to understand the potential for mycorrhizae to impose selection on orchid phenotypes would also be useful.

4.2. Concordance between morphological differentiation and genetic differentiation

Whereas previous studies identified significant morphological and genetic divergence in *P. dilatata* at broad geographic scales, they have not previously correlated genetic differentiation with phenotypic divisions (Wallace, 2003a; Adhikari and Wallace, 2014). The allelic variation reported in this study provides the strongest indication yet that a shared evolutionary history connects phenotypically similar populations and distinguishes these from phenotypically dissimilar populations. The genetic dataset has also revealed some major differences between the phenotypic groups. For example, small-flowered populations that are not strongly admixed have lower allelic variation and heterozygosity and greater population differentiation compared to large-flowered populations. These results suggest greater isolation, which could occur due to lower density of populations and reduced gene flow over widely spaced muskeg habitats, as non-forest habitats cover only 17% of the landscape in Southeast Alaska.

The deep genetic divergence in phenotypic groups may also reflect historical divergence, perhaps associated with Pleistocene refugia in this area (Carrara et al., 2007; Marr et al., 2008; Geml et al., 2010; Shafer et al., 2010). The Alexander Archipelago of Southeast Alaska contains more than 2,000 individual islands and stretches across 16,000 km of coastline (Carrara et al., 2007), giving the region's extensive topographical and geographical complexity that undoubtedly influences gene flow and population isolation. The impact of the last glacial period was heterogenous across Southeast Alaska, with numerous refugia proposed along the western edges of the Alexander Archipelago and exposed areas of the continental

shelf (Carrara et al., 2007). More recently, successional changes in coastal vegetation were associated with uplift following the Little Ice Age between 1770–1790 (Motyka, 2003). With isostatic changes continuing to occur, extensive uplift meadows may have rapidly developed in the area (e.g., Auffret and Cousins, 2018). While these habitats could represent an earlier successional stage relative to muskeg, they may harbor older genetic lineages if they persisted during glaciation. Phylogeographic studies would be useful to understand the evolutionary and historical connections among populations in the study area and the presence of multiple refugia within the Alexander Archipelago or dispersal from other refugia in northwestern North America.

4.3. Hybridization between divergent phenotypes

While most populations we studied have at least one admixed sample (Figures 4A,B), the extensive and cryptic introgression that characterized several small-flowered populations was unexpected as these populations are morphologically similar to other small-flowered populations sampled in muskeg bogs. While other studies have reported cryptic introgression, for example in *Protea* L. (Mitchell and Holsinger, 2018) and in *Lomatia* R. Br. (McIntosh et al., 2014), these studies also found hybrids with both genetic and morphological intermediacy. In our study, morphologically intermediate populations were not readily detected when averaged across samples. Nevertheless, at the individual level, statistical outliers representing larger flowers were observed in muskeg populations and could indicate admixed individuals due to pollinator-mediated gene flow from large-flowered populations (M.L. Bowles, unpublished data).

Phillips et al. (2012) suggested that seed dispersal between populations at regional scales (e.g., < 250 km) is likely common, but gene flow might be more limited at larger geographic scales. Our analyzes indicated that genetic distance among populations reflects isolation by distance at large scales but not at small scales, consistent with the patterns described by Phillips et al. (2012). The low incidence of admixture detected by STRUCUTRE and NewHybrids in the four hybrid populations suggests that introgression may have occurred swiftly and early in their history. Given the commonality of small-flowered populations in muskeg, we suggest it is more likely that these small-flowered populations were colonized from other small-flowered populations, rather than large-flowered populations. If gene flow occurred early in their establishment and was not maladaptive, then it would persist in the growing population. The alternative explanation for the genetic similarity of hybrid populations to large-flowered populations, that they originated from large-flowered colonizers of muskeg bogs that subsequently evolved smaller flowers, seems less likely in the absence of a functional basis for variation in flower size due to climate or soils.

The contemporary presence of large-flowered populations in more diverse habitats may indicate greater historical abundance across the landscape compared to small-flowered populations and muskeg habitats (Auffret and Cousins, 2018). To produce extensive and cryptic introgression in the small-flowered populations, large-flowered plants would need to be nearby and accessible to pollinators to facilitate repeated introgression and backcrossing with newly colonized small-flowered populations in the area. All but one of the hybrid populations sampled (GI) are located within 250 km of a large-flowered population in the study area, which is consistent with the maximum distance for seed dispersal that was suggested by Phillips et al. (2012).

While seed dispersal may have led to colonization of small-flowered forms in areas containing large-flowered populations, pollinators must be the agents of gene flow leading to introgression. Different spur lengths are expected to reduce cross-pollination between phenotypes, but not to prevent it. Because hawkmoths are strong fliers that may easily cross between habitats, gene flow may be more easily mediated from larger flowers to smaller flowers. Noctuidae moths can also transport pollen long distances (Hendrix et al., 1987), but this may be more likely during migration. Whereas pollinaria adhere to the proboscis of both pollinators, they would adhere closer to the eyes of Noctuidae moths visiting longer-spurred flowers of *P. dilatata* (Figure 3) than for hawkmoths. In short-spurred flowers, positioning of pollinaria closer to the proboscis tip for hawkmoths might facilitate contact with the column leading to successful cross-pollination. Thus, even occasional visits to these populations by hawkmoths carrying pollinia from long-spurred populations could have long-lasting impacts because an orchid pollinarium contains enough pollen to fertilize thousands of ovules. Many inter-specific hybrids are known within *Platanthera* (Wallace, 2003b; Brown, 2004; Alcantara et al., 2006; Brown et al., 2008; Wettewa et al., 2020; Hartvig et al., 2022), indicating that spur length does not consistently prevent cross-pollination and pollinators readily move pollen between species.

4.4. Genetic structure and factors influencing gene flow

Factors determining genetic structure may vary across the landscape and across spatial scales. We expected that across the extent of the study area, which is nearly 500 km, geographic distance would be important because of limited gene flow. By contrast, within the areas where seed dispersal can occur over shorter distances or pollinators are capable of flying between sites, environmental factors are expected to be more important determinants of genetic structure. In the study area, both elevation and habitat differences might influence gene flow at varying scales. When considering all populations, both geographic distance and habitat (i.e., elevation, soils, and climate) are significant predictors of genetic distance. Nevertheless, consistent with our hypothesis, geographic distance explained more of the observed variation in genetic distances than environmental factors did (Table 3; Supplementary Figure S2). The extensive topographic variation of Southeast Alaska could impose barriers to gene flow if orchid seeds are not able to move between mountains and the orchids are adapted to soil types or interact with other organisms, e.g., mycorrhizae or pollinators, that are themselves restricted by environmental factors.

At a smaller spatial scale encompassing the central populations, *ca.* 50 km north-to-south, we found that geographic distance was not predictive of genetic distance. This suggests that seeds and/or pollinators readily move about populations at this scale. By contrast,

environmental factors were found to significantly influence genetic distance, indicating the presence of habitat barriers to successful movement. This is expected if the small and large-flowered populations are adapted to different habitats or are limited by symbiotic partners that are themselves adapted to these differing habitats as noted for larger geographic scales. The difference in the pattern of genetic structure across spatial scales demonstrates a complexity of landscapes in how they influence population connectivity. While these results suggest that orchid seeds and or pollen may readily move about, we are unable to discern the relative importance of these factors for gene flow. Additionally, the complex history of this region has undoubtedly impacted the patterns observed today, but without a phylogeographic context we also cannot account for how historical factors have influenced the genetic structure of *P. dilatata* in this region. Future studies that test hypotheses about the locations of glacial refugia are important foci for future studies of this species across western North America.

4.5. Taxonomic implications

With four habitats and two phenotypic groups, eight unique combinations could characterize *P. dilatata* in Southeast Alaska. Yet, we found only five of these combinations as small-flowered plants are restricted to muskeg bogs and large-flowered plants are rarely found in these habitats. The deep genetic divergence between groups of populations supports the inference that there are multiple evolutionary lineages in the study area. Nevertheless, placing these lineages within the current taxonomy of this species is difficult. Plants from Southeast Alaska have a mean spur length that exceeds lip length (Figure 3A), which would place all of them in var. *leucostachys*. Yet, the range of spur and lip lengths measured on plants in the study area (spurs: 6.5–9 mm for small-flowered and 9–12 mm for large-flowered; lips: 5.25–7.25 mm for small-flowered and 7–9.5 mm for large-flowered) encompasses or exceeds the lengths described for the three varieties by previous authors (Sheviak, 2002; Wallace, 2003a; Sears, 2008; Adhikari and Wallace, 2014; Supplementary Table S6) but lie primarily within vars. *dilatata* and *leucostachys*. These morphological measurements are not consistent with varietal circumscriptions by Sheviak (2002) or the suggestion that three varieties occur in Southeast Alaska.

An additional consideration in metric comparisons among studies is the presence of artifacts associated with measurement methods. It is difficult to measure nectar spur length because they are falcate; thus, intact spurs will appear shorter than flattened spurs. The source of the flowers for measurement (i.e., fresh, dried, or spirit-preserved) also influences measurements as preservation can introduce distortions (Bateman et al., 2013), and spur length has been reported to increase over the flowering season in individual plants (Sheviak, 2002).

Taxonomic revision of *P. dilatata* is warranted because the division of three varieties is inadequate to explain the variation encountered in many areas of the distribution. Furthermore, ecological or pollination studies should explicitly include morphological measurements of samples, rather than simply giving a varietal designation, as this would provide more transparency in morphological variability of studied populations. Such data would also contribute to a greater ability to synthesize variation at local scales, which is needed to evaluate the cohesiveness of *P. dilatata* populations and to quantify the geographic scale of discord in morphological and genetic divergence.

5. Conclusion

By studying genetic, morphological and habitat diversity at the regional scale in *P. dilatata* we have identified novel patterns, yet consistency with previous studies on this species. Strong genetic divergence between flower groups suggests the presence of distinct evolutionary lineages within Southeast Alaska. Evidence of bidirectional gene flow between flower forms, nevertheless, indicates that they are not reproductively isolated. Although orchid seeds are considered capable of long-distance gene flow, our results indicate that gene flow most readily occurs only at shorter geographic distances, perhaps <50 km. Environmental factors also contribute significantly to genetic structure and could reflect adaptations of the orchids themselves to these habitats or adaptations of their symbiotic partners. Further studies are needed to understand the evolution of adaptation in this species and its phylogeographic history. *Platanthera dilatata* should be considered a model system for understanding the process of diversification in temperate orchids.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary files, further inquiries can be directed to the corresponding author.

Author contributions

LW and MB conceived of the study, collected data, wrote the manuscript, and critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1085938/full#supplementary-material

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Diversity and specificity of orchid mycorrhizal fungi in a leafless epiphytic orchid, *Dendrophylax lindenii* and the potential role of fungi in shaping its fine-scale distribution

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Orchids grow in diverse habitats worldwide with most (approximately 69%) growing on trees as epiphytes. Although orchid mycorrhizal fungi have been identified as potential drivers for terrestrial orchid distribution, the influence of these fungi on the fine-scale distribution of epiphytic orchids is poorly understood. In this study, we investigated the mycorrhizal fungal community and fine-scale distribution of *Dendrophylax lindenii*, a rare and endangered epiphytic orchid that is leafless when mature. We used amplicon sequencing to investigate the composition of orchid mycorrhizal fungi in the roots of 39 *D. lindenii* individuals in their natural habitat, the swamps of Florida. We compared the orchid mycorrhizal fungi of *D. lindenii* to those of co-occurring epiphytic orchids, as well as to the orchid mycorrhizal fungal communities of bark from potential host trees, with and without *D. lindenii*. Our results show that *D. lindenii* has a high specificity for a single *Ceratobasidium* species, which is widely distributed on phorophytes and detected in both wet and dry periods in the orchid's habitat. This *Ceratobasidium* species was mostly absent or only recorded in low frequency in the roots of co-occurring epiphytic orchids. Phylogenetic analysis documented that this *Ceratobasidium* was conspecific with the strain that is used to germinate *D. lindenii ex-situ*. However, our findings suggest that laboratory germinated adult *D. lindenii* transplanted into the field had lower read abundances of this *Ceratobasidium* compared to naturally occurring plants. These findings suggest that this orchid mycorrhizal fungus may play a significant role in the fine-scale distribution of naturally occurring *D. lindenii*.

KEYWORDS

conservation, *Ceratobasidium*, host tree specificity, amplicon sequencing, ghost orchid

Introduction

Mycorrhizal fungi are well known mutualists that are essential for their plant partners' abundance and spatial distribution (Smith and Read, 2010; McCormick and Jacquemyn, 2014). While *ca.* 69% of orchid species are tropical epiphytes (Zotz, 2016), little is known about the orchid mycorrhizal fungi (OMF) they associate with compared to temperate terrestrial orchids.

Epiphytic orchids, like their terrestrial counterparts, enlist OMF to facilitate seed germination and seedling development, but it remains unclear to what degree epiphytes continue to utilize OMF into maturity (Dearnaley et al., 2012; Rasmussen et al., 2015; Selosse et al., 2022). Stable isotope work by Gebauer et al. (2016) revealed that a greater number than previously thought of orchids are likely reliant on OMF, and are functioning as myco-heterotrophs even though they are photosynthetic as adults. This finding of likely orchid dependence on OMF as adults, especially epiphytic orchids, raises the question of the potential role that OMF play in driving their fine-scale spatial distribution.

The drivers of fine scale epiphyte spatial distribution and host tree (phorophyte) specificity have been debated within the literature for over a century since the writings of Schimper (1888) see review by Wagner et al. (2015). Debate has focused on the role of various abiotic factors (e.g., microclimate and host bark characteristics) and biotic factors (e.g., symbiotic fungi and co-occurrence with moss). Research by McCormick et al. (2018) has demonstrated that while OMF may restrict terrestrial orchid distributions at local scales, at broad geographic scales terrestrial orchids are not constrained by OMF. Most of these findings were established for terrestrial orchids, with investigations of epiphytic orchids still pending (Li et al., 2021). Recently, studies have investigated fungal communities in the bark of phorophytes of epiphytic orchids, which providing insights into phorophyte specificity and spatial distribution of epiphytic orchids (Izuddin et al., 2019; Eskov et al., 2020; Pecoraro et al., 2021; Petrolli et al., 2021, 2022). Eskov et al. (2020) further explored OMF and revealed that fungi colonizing epiphytic orchid roots were significantly different from the phorophytes' branches. Pecoraro et al. (2021) studied the phorophyte specificity of two epiphytic orchid species, as well as the environmental factors influencing the relationship between the orchids and their phorophytes. They concluded that the orchid phorophyte associations were influenced by the phorophyte bark's OMF communities and potentially its pH and water holding capacity. Recent studies have also revealed examples of a strong fungal specificity of epiphytic orchids associated with a single OMF species, *Ceratobasidium* or Tulasnellaceae species (Rammitsu et al., 2019, 2020) despite inconclusive early studies (Gowland et al., 2013; Wang et al., 2017).

Amplicon sequencing, a type of environmental sequencing, is a cost-effective advancement for investigating fungal communities compared to traditional culture-based methods (McCormick and Jacquemyn, 2014; McCormick et al., 2018). Ectomycorrhizal (ECM) fungi as well as some OMF are known to be recalcitrant to being cultured and recent studies utilizing amplicon sequencing have detected a diversity of ECM fungi in the roots of both epiphytic and terrestrial orchids (Selosse et al., 2022). Additionally, amplicon sequencing can increase the detection of potential OMF in epiphytic orchid roots compared to Sanger Sequencing as Sanger Sequencing is often limited by the need for first culturing the fungi (Waud et al., 2016; Jacquemyn et al., 2017; Novotná et al., 2018; Johnson et al., 2021).

We chose the rare leafless epiphytic orchid *Dendrophylax lindenii* (Lindl.) Bentham ex Rolfe (Figure 1) as our study taxon to further document OMF communities of rare tropical epiphytic orchids and to examine the potential role of OMF as drivers of their phorophyte specificity. In addition to sampling the roots of *D. lindenii* we sampled co-occurring epiphytic orchids and the bark of potential phorophytes with and without *D. lindenii* to uncover evidence of OMF specificity during two periods, flooded and not flooded in the area of its natural distribution in the United States (Supplementary Figures S1, S2).

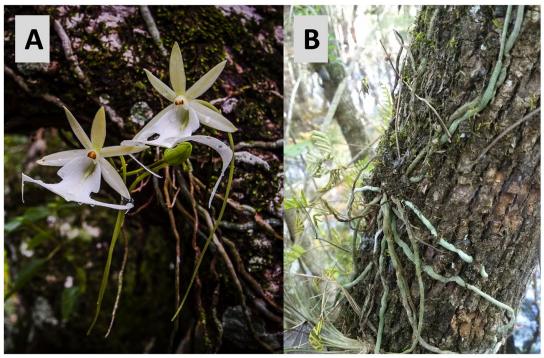

FIGURE 1
(A) Flowers of *Dendrophylax lindenii* (photo by Larry W. Richardson). **(B)** *Dendrophylax lindenii* roots growing on the tree trunk of a phorophyte, *Fraxinus caroliniana*.

Dendrophylax lindenii, also known as the Ghost Orchid, is restricted to southwestern Florida and the western tip of Cuba (Brown, 2002) where it remains vulnerable to poaching and environmental changes (Mújica et al., 2018, 2021). In Florida, less than 1,500 individuals are thought to remain (Haaland et al., 2022), and in Cuba, the number is even fewer [<500; (Mújica et al., 2018)]. In the Florida Panther National Wildlife Refuge where about 1/3rd of the state's Ghost Orchids are found, Mújica et al. (2018) calculated that *D. lindenii* numbers will decline by 20% during the next decade. Consequently, the species is now a candidate for U.S. Federal protection under the Endangered Species Act (Haaland et al., 2022). The Florida habitats of *D. lindenii* consist of cypress domes and strand swamps in the Big Cypress Basin. According to a study by Mújica et al. (2018) in 2015, 69% of the growth of *D. lindenii* in Florida is found on the trunks of *Fraxinus caroliniana* Mill., while occurring less frequently (36%) on *Annona glabra* L. These trees are typically located in the lower canopy under *Taxodium distichum* (L.) Rich. (Brown, 2002; Stewart and Richardson, 2008). Although *D. lindenii* grows in a moist habitat, it experiences dry periods during the region's dry season which lacks any standing water (Mújica et al., 2018).

Like all orchids, *D. lindenii* requires OMF for germination (Hoang et al., 2017). Early seedling stages of *D. lindenii* have a rudimentary ephemeral leaf. As an adult, the orchid lacks leaves and shoots and photosynthesizes predominantly via its roots (Benzing and Ott, 1981; Benzing et al., 1983; Hoang et al., 2017). Benzing and Ott (1981), have shown that the mature roots of *D. lindenii* utilizes CAM photosynthesis, and Chomicki et al. (2014) using microscopy hypothesized that it forms a mutualism with an OMF (Ceratobasidiaceae) to obtain carbon to supplement its photosynthesis. Furthermore, seed germination experiments by Hoang et al. (2017) and Mújica et al. (2018) have confirmed that *D. lindenii* associates with a *Ceratobasidium* and that this fungus is present in mature roots.

Our primary aim for this study was to identify the OMF associated with *D. lindenii* and to investigate the potential role of OMF in influencing its fine-scale distribution within naturally occurring populations (i.e., why it was found on some potential phorophytes and not on others). We tested two hypotheses: (1) *D. lindenii* has a specific community of OMF compared to co-occurring epiphytic orchids; and

(2) the OMF colonizing *D. lindenii* are found in the bark of *D. lindenii* phorophytes in higher abundances than in the bark of trees without *D. lindenii*. Given that *D. lindenii* is currently state-listed as endangered, we primarily restricted our sampling to root tips to minimize damage to the plant. To investigate if additional OMF were missed with this sampling method we also investigated the fungal community of four whole roots.

Materials and methods

Study sites, tree bark and orchid root sampling

During 2016 and 2018 we collected >100 root and bark samples from five sites at the Florida Panther National Wildlife Refuge (FPNWR) a 10,684 ha area (Supplementary Table S1). Four of the sites were natural habitats for *D. lindenii*. The fifth site lacked naturally occurring plants but had *D. lindenii* explants that were micropropagated under axenic conditions in the lab and subsequently transplanted (attached) on appropriate species of trees. The site with explants we identified as Site 4 in our study. Most of the sites were dominated (over 90%) by *F. caroliniana* as the main phorophyte. Sites were either sloughs or strand swamps and were separated by about 1 km from each other. When we collected samples in 2016 (March), FPNWR sites all had standing water in sloughs and swamps (Supplementary Figure S1), but all sites were dry (not flooded) when we sampled in 2018 (April) (Supplementary Figure S2). This sampling period in 2018 was unusually dry. The precise sites at the FPNWR are not disclosed herein because *D. lindenii* and several co-occurring orchids are state-listed as endangered and remain highly vulnerable to poaching. For each site, Special Use collecting permits were obtained (USFWS, OMB Control # 1018–0102), and permission to access and sample *D. lindenii* populations was subsequently granted.

In March 2016, root samples were collected from four sites at the FPNWR Sites 1–4 (Supplementary Table S1). Root samples were collected from the leafless epiphytic orchid species *D. lindenii* ($n = 9$) and several co-occurring epiphytic orchids: *Campylocentrum pachyrrhizum* (Rchb.f.) Rolfe ($n = 3$), *Dendrophylax porrectus* (Rchb.f.) Carlsward & Whitten ($n = 6$), *Epidendrum amphistomum* A. Rich. ($n = 4$), *Epidendrum nocturnum* Jacq. ($n = 1$) and *Prosthechea cochleata* (L.) W. E. Higgins ($n = 3$). Simultaneous with the collection of root samples, bark samples were collected from phorophytes adjacent of all epiphytic orchids (Supplementary Table S1).

In April 2018, sampling of the roots of an additional 27 *D. lindenii* plants was carried out at the original four sites plus one additional site (Site 5). Concurrent with the root tissue collection, bark samples ($n = 57$) were collected from phorophytes of *D. lindenii* and trees without *D. lindenii* (Supplementary Table S1). Five trees with and five trees without *D. lindenii* individuals were sampled at each of the five sites. The sampling design considered the position of *D. lindenii* on the tree and bark samples were collected from (1) the base of the tree trunk, (2) above *D. lindenii*, (3) the side of roots of *D. lindenii* root; and (4) the opposite side of the tree trunk (Supplementary Figure S3). In instances where *D. lindenii* was not present bark samples were collected from the base of the tree and three additional samples were taken at a height of at breast height (1.5 M) from base, where *D. lindenii* would typically grow.

Additionally, we conducted a pilot study to assess the success of amplicon sequencing of root tips for revealing the OMF community of *D. lindenii*. We obtained 50 mm root samples collected from three mature individuals of *D. lindenii* at the FPNWR. A root of a *D. lindenii* that was home cultivated from Redlands, Florida was also sampled. For this pilot study, roots were cut into 5 mm long segments starting from the tip and labelled alphabetically (i.e., A, B, C, etc. see Supplementary Figure S4).

DNA extraction, PCR amplification, and amplicon sequencing

Approximately 5 mm of root tip and bark tissue was collected and stored in cetyltrimethylammonium bromide (CTAB) buffer. Root and bark samples were surface sterilized with 70% ethanol, and 50% Clorox® (2.6% sodium hypochlorite) using the method outlined in Bayman et al. (1997). Next, genomic DNA was extracted from root samples using the Qiagen DNeasy extraction kits (Qiagen, Valencia, CA, United States) following the manufacturer's instructions. DNA from bark samples was extracted with the modified CTAB method of Murray and Thompson (1980), and for difficult to extract samples the MOBIO Power Soil DNA Extraction kit (MOBIO Laboratories, Carlsbad, CA, United States) was used following the manufacturer's instruction.

The extracted genomic DNA from the 2016 root samples was amplified using the primers: ITS86f (5′- GTGAATCATCGAA TCTTTGAA-3′; Turenne et al., 1999) and ITS4 (5′- TCCTCCGCT TATTGATATGC-3′; White et al., 1990). These fungal primers (ITS86F/ITS4) amplify the internal transcribed region ITS, the standard fungal barcode, for ITS subregion 2 which is shown to be effective for delimiting OMF such as those in the Cantharellales.

Next, amplicons from the PCR products were produced using a three step PCR sequencing protocol (see Johnson et al., 2021 materials and methods). This included PCR steps that used modified primers with indices from the Nextera XT kit for 96 indices to sequence 2 × 250 bp. The final amplicon libraries generated for root and bark samples were quantified using a Qubit dsDNA HS kit (Invitrogen) and a Bioanalyzer-Agilent 2100 (Agilent Technologies, Santa Clara, CA, United States). Final amplicon libraries for root and bark samples were pooled together in equimolar concentrations and the final pool was then sequenced on an Illumina MiSeq at the Pritzker Lab at the Field Museum (Chicago, IL).

The root sections from the pilot study, root tips, and bark samples from 2018 were PCR amplified using modified fungal primers ITS86F/ITS4 with barcodes supplied from Novogene Bioinformatics Institute (Beijing, China) following the protocol applied in 2016. The generated final amplicon libraries were pooled to equimolar concentrations then shipped to Novogene and sequenced on an Illumina HiSeq. Sequences generated from this study were submitted to NCBI's Sequence Read Archive under the BioProject PRJNA948888.

Bioinformatics and statistical analyses

Initially, bioinformatics analyses were performed on roots and bark collected in 2016 separately. Subsequently, the sequences obtained from the bark, root, and root sections of the 2018

dataset were integrated, and bioinformatics analyses were conducted on these samples collectively to determine patterns of similar Operational Taxonomic Units (OTUs) between sample types.

To conduct bioinformatic analyses, the sequences were first quality filtered, followed by OTU clustering utilizing the PIPITS pipeline (version 1.4.0) default settings as described by Gweon et al. (2015). Briefly, PIPITS joined reads and quality filtered short reads (<50 bp), extracted non ITS fungal reads with the script ITSx (Bengtsson-Palme et al., 2013), then clustered OTUs at 95% sequence similarity. Additional PIPITS scripts assigned taxonomy to OTUs with the Ribosomal Database Project Classifier [a Naïve Bayesian Classifier (Wang et al., 2007)] and the UNITE database (Nilsson et al., 2019). Sequences for the HiSeq dataset was analyzed separately from the 2016 MiSeq dataset. The single difference between analysis of the MiSeq data analyses and HiSeq data analyses was omitting the ITSx step for the HiSeq data.

To further investigate differences between fungal communities we filtered rare OTUs that were less than 1,000 sequences, and the raw read abundances were then normalized with Cumulative Sum Scaling in the R package metagenomeSeq (Paulson et al., 2013). All statistical analyses were conducted within R (R Core Team, 2022). The visualization of abundance of sequences was first accomplished using Krona charts, which were generated using Krona-2.8.1 within R (Ondov et al., 2011). Bar graphs showing relative and read abundances were produced with the R package ggplot2. To better visualize differences between read abundances, the y-axis was truncated using the R package ggbreak (Xu et al., 2021).

Principal coordinate analysis (PCoA) was generated using Bray-Curtis distances with the R package vegan and visualized with ggplot2 (Wickham, 2016). Significance between fungal communities of *D. lindenii* and epiphytic orchid roots, phorophyte and the bark of trees without *D. lindenii* present, and location of sites were determined with "permutational manova" (Anderson, 2001) in R package vegan (adonis2 function) by first permuting the raw data with 9,999 permutations (Oksanen et al., 2022). Prior to executing adonis2 for the permutational multivariate analysis of variance (PERMANOVA) we also investigated the dispersion for groups using another vegan function betadisper. In addition, pairwise comparisons were completed for the PERMANOVA using the pairwiseAdonis R package with Bonferroni corrections (Martinez Arbizu, 2017). p-values that are < 0.05 were considered significant.

Phylogenetic analyses were undertaken to investigate relationships among the community of recovered Ceratobasidiaceae sequences from 2016 and 2018 root and bark fungal samples. The phylogenetic tree incorporated *Ceratobasidium* sequences from NCBI GenBank. A sequence of *Tulasnella* from the UNITE fungal database was used as an outgroup. We used MUSCLE (Edgar, 2004) in AliView version 1.27 (Larsson, 2014) for multiple sequence alignments and also used AliView to generate a Maximum Likelihood tree using the default settings of the program FastTree version 2.1.10 (Price et al., 2009). The final tree was rooted and visualized using FigTree version 1.4.4.[1]

1 http://tree.bio.ed.ac.uk/software/figtree/

Results

Sequence analyses of root and bark samples

Fungal sequence data were obtained from roots of *D. lindenii* and other co-occurring epiphytic orchids collected in the field in 2016 and 2018. Root samples in 2016 and 2018 yielded 537,371 ($n = 26$) and 1,691,086 reads ($n = 30$), respectively, resulting in the identification of 526 and 1,077 Operational Taxonomic Units (OTUs) at the 95% sequence identity level. Sequences generated from sections of whole roots yielded 3,205,959 reads ($n = 37$ root section samples) and resulted in the identification of 1,372 OTUs. In 2016, we collected 30 bark samples and sequencing yielded 693,482 reads with a total of 550 OTUs. Most phorophytes sampled in 2016 were from *F. caroliniana* (over 90%) with a small proportion of *A. glabra* also being sampled (Supplementary Table S1). Additionally, a bark sample was collected from a *Taxodium distichum* that had an explant affixed to it (Supplementary Table S1). In 2018 we successfully sequenced 57 bark samples mostly from *F. caroliniana*, from trees with *D. lindenii* ($n = 43$) and trees without *D. lindenii* ($n = 14$), yielding 7,245,995 reads with a total of 1,141 OTUs resolved.

The increase in read and OTU counts in 2018 can be attributed to the use of the HiSeq platform instead of the MiSeq platform, as well as the greater sampling intensity of root samples. The average OTU richness observed in root and bark samples for 2016 was 67 and 55 respectively, whereas the OTU richness for 2018 samples for roots was 233 and 304 for bark (Supplementary Table S1). Unfortunately, no amplicon libraries were generated for root samples collected at Site 2 in 2016 as the library preps were unsuccessful resulting in sequence data that was unsuitable for data analysis. The analysis of the pilot study examining potential differences in fungal communities in different sections of entire roots of both cultivated and wild collected *D. lindenii* documented that fungal communities were similar across all sections but were different between cultivated vs. wild collected plants (Supplementary Figure S5).

Ceratobasidiaceae is the dominant OMF associated with *Dendrophylax lindenii*

The fungal communities of naturally occurring *D. lindenii* roots across all sites were observed to be similar and dominated by several Ceratobasidiaceae, even during the flooded (2016) (Figure 2; Supplementary Figure S6A) and not flooded (2018) periods (Supplementary Figure S6B). The dominant Ceratobasidiaceae OTUs associated with *D. lindenii* were OTU 11 (recovered from bark samples), OTU 14 (recovered from 2016 root samples); and OTU 76 (recovered from both 2018 root and bark samples). Phylogenetic analysis resolved each of these dominant *Ceratobasidium* OTUs as part of a well-supported monophyletic clade, Clade 2, and are considered conspecific (Figure 3). A visual analysis of sequence alignments further supports this finding, as Clade 2 OTUs exhibit a sequence similarity of more than 98%. *Ceratobasidium* Clade 2 includes other individuals that were previously recovered from mature roots of *D. lindenii*. Included in Clade 2 is Dlin-394, which was derived from cultures isolated from mature roots of *D. lindenii* and has been used to germinate seeds of *D. lindenii* (Hoang et al., 2017).

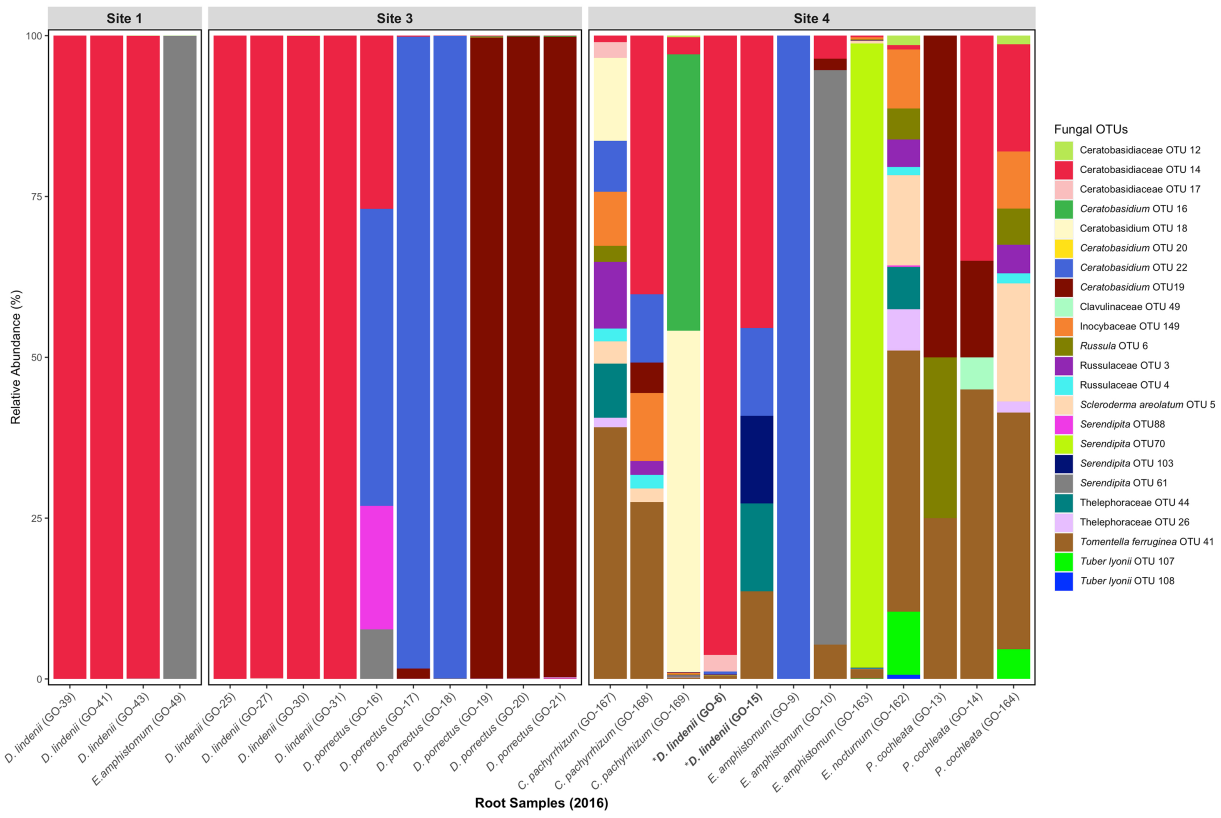

FIGURE 2
Relative abundance of fungal OTUs (putative OMF) obtained from root samples of *D. lindenii* and co-occurring epiphytic orchids in 2016. *Dendrophylax lindenii* explants at site 4 are bolded and are labeled with an asterisk.

The pilot study undertaken to assess if the OMF community recovered from root tips of *D. lindenii* provided was reflective of the full root OMF community provided further evidence of the dominance of *Ceratobasidium* Clade 2 in naturally occurring *D. lindenii*. The majority of root sections from naturally occurring *D. lindenii*, including the root tips, were dominated by *Ceratobasidium* Clade 2 (Supplementary Figure S5). Of note, root section samples of the home-cultivated *D. lindenii* lacked Ceratobasidiaceae OTUs. Instead, an abundance of Ascomycota OTU reads (*Lasiodiplodia* OTU 1174 *and Diaporthales* OTU 627) were recovered (Supplementary Figure S5).

Ceratobasidium Clade 2 was present in most samples of *D. lindenii* (Supplementary Figure S6C); however, it was absent or only had very low read numbers from the roots of co-occurring epiphytic orchids (Figure 2). Other taxa of Ceratobasidiaceae were recovered from these orchids, e.g., Ceratobasidiaceae OTUs 19 and 22 were abundant in root samples of *D. porrectus* (Figure 2). These OTUs belonged to different clades (Clade 1 and 3, Figure 3). Other OMF taxa that were recovered from co-occurring epiphytic orchid roots collected in 2016 (Figure 2; Supplementary Figures S6A,B) included taxa of putative OMF Serendipitaceae and ECM fungi such as Inocybaceae, Russulaceae, *Scleroderma*, Thelephoraceae, *Tomentella*, and *Tuber* species. While present in lower proportions (<1%) we did not detect a widespread presence of Tulasnellaceae OTUs, a traditional OMF.

Diversity of OMF in bark of *Dendrophylax lindenii* phorophytes and trees without *Dendrophylax lindenii*

Unlike the root fungal community, the bark fungal community was not dominated by *Ceratobasidium* Clade 2 or other Ceratobasidiaceae OTUs. Ceratobasidiaceae OTUs accounted for less than 5% of the total reads (Supplementary Figures S7A,B). Nonetheless, *Ceratobasidium* Clade 2 was present in most bark samples (Figure 4). Other putative OMF detected in bark samples were also rare and included a few Serendipitaceae and *Tulasnella* OTUs. Additional rare OTUs also included ECM fungi including *Mycena*, *Russula*, Thelephoraceae, and *Tomentella*.

The principal coordinate analysis (PCoA) and subsequent PERMANOVA tests on root samples collected in both 2016 and 2018 showed significant differences between sites and orchid species (Supplementary Figures S8A,B). Specifically, the 2016 root samples reveal potentially significant differences in both orchid species (PERMANOVA: $F_{5,25} = 2.11$, $R^2 = 0.29$, $p < 0.05$, betadisper: $F = 2.08$, $p = 0.11$) and location (PERMANOVA: $F_{2,25} = 3.20$, $R^2 = 0.18$, $p < 0.05$, betadisper: $F = 5.09$, $p = 0.01$). Furthermore, pairwise comparisons showed significant differences between Site 4 (site with only introduced *D. lindenii* lab grown explants) and the two other sites, Site 1 (adjusted $p = 0.009$) and Site 3 (adjusted $p = 0.003$). In addition, Site 4 also differed from Site 3 (adjusted

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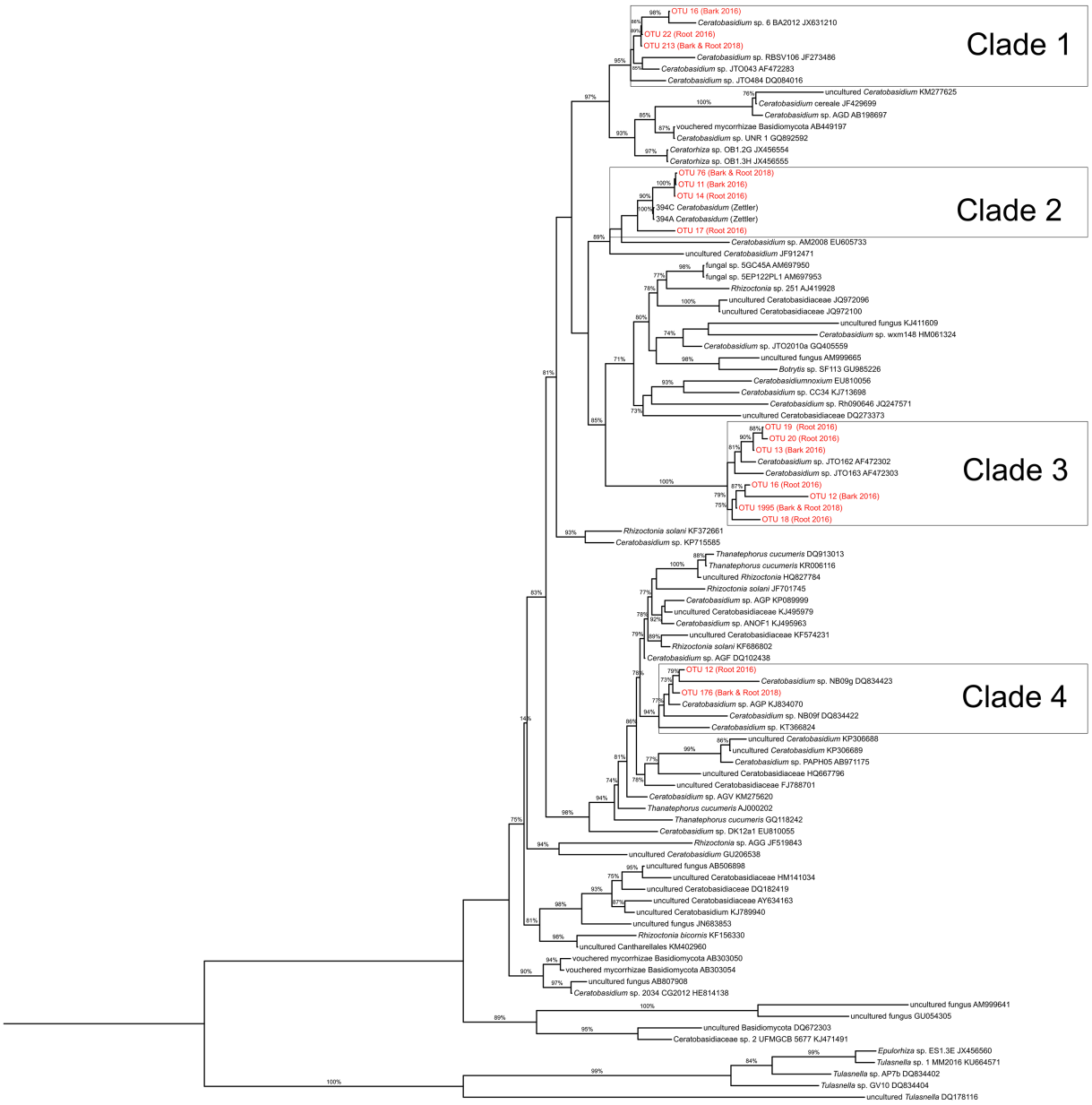

FIGURE 3
Maximum likelihood phylogeny of putative Ceratobasidiaceae species based on ITS data set of 95 taxa using FastTree constructed with default parameters. A cultured *Tulasnella* was used as an outgroup taxon. Bootstrap support values above 70% are reported. Sequences generated during this study are indicated in red.

$p = 0.03$). Similarly, the *D. lindenii* root samples collected in 2018 revealed significant differences by site (PERMANOVA: $F_{4,24} = 1.26$, $R^2 = 0.20$, $p = 0.036$, betadisper: $F = 1.24$, $p > 0.5$). However, pairwise comparisons revealed no significant differences between sites when adjusted p values were generated.

PCoA of bark data collected for 2016 (Supplementary Figure S9A) revealed significant differences for both location (PERMANOVA: $F_{4,34} = 1.82$, $R^2 = 0.16$, $p < 0.05$, betadisper: $F = 1.47$, $p > 0.05$) and the presence of *D. lindenii* (PERMANOVA: $F_{4,34} = 1.50$, $R^2 = 0.03$, $p = 0.028$, betadisper: $F = 0.69$, $p = 0.4$). Although sites were not different during the flooded period of 2016, pairwise comparisons of the 2018 bark data (corresponding PCoA is Supplementary Figure S9B) revealed differences between Site 1 and Site 3 (adjusted $p = 0.01$); differences between Site 2 and Site 5 (adjusted $p = 0.03$); and differences between Site 2 and Site 4 (adjusted $p = 0.05$).

Discussion

Our study provides strong evidence that *D. lindenii* may have a high specificity for a single Ceratobasidiaceae OTU (*Ceratobasidium* Clade 2) in its natural habitat at the Florida

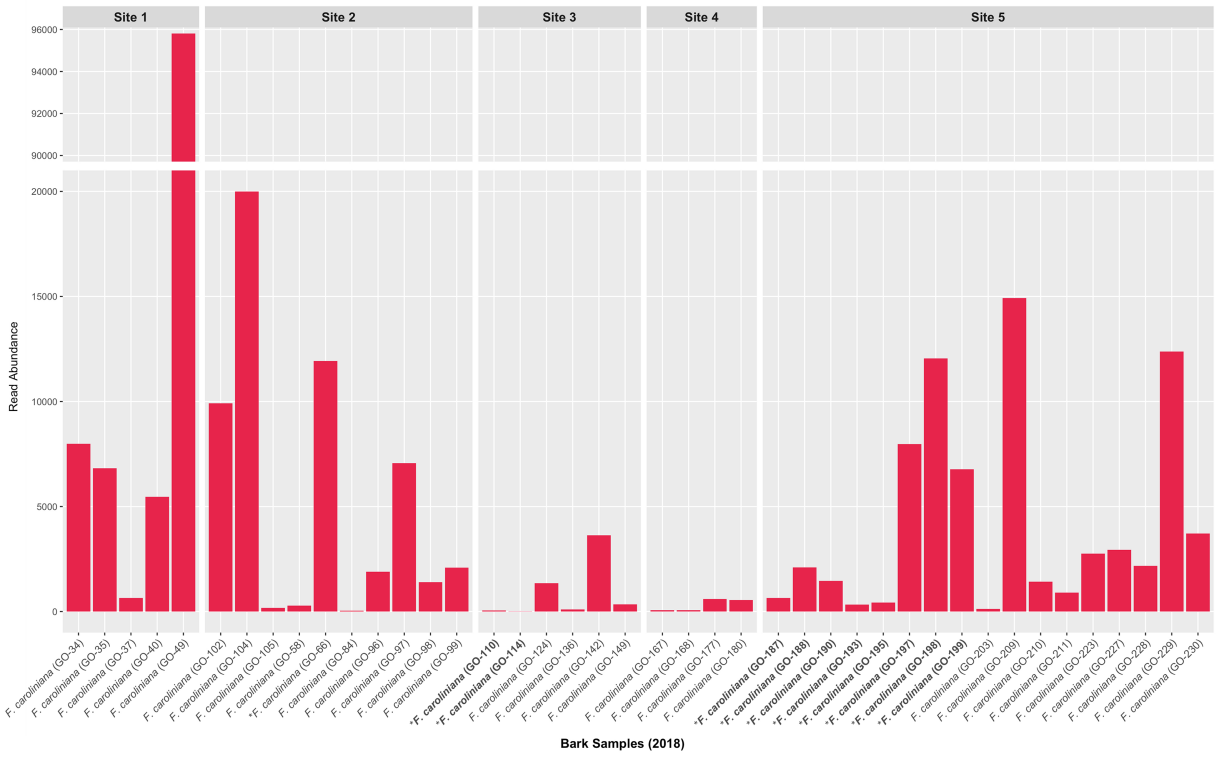

FIGURE 4
Read abundance of *Ceratobasidium* clade 2 obtained from bark collected in 2018 from trees with and without naturally occurring *D. lindenii*. Bar graph is condensed to better visualize the sample with the highest read abundance. Trees without naturally occurring *D. lindenii* are represented in bold and have an asterisk. All bark samples shown in graph are from *F. caroliniana* obtained at various positions on the trunk except the base (see Supplementary Table S1 for bark sample positions).

Panther National Wildlife Refuge (FPNWR). This OTU was found to be abundant in *D. lindenii* roots, and rare (<1% of total reads) in other co-occurring epiphytic orchids at the FPNWR. *Ceratobasidium* Clade 2 was also widespread at all sites in the bark of phorophytes with *D. lindenii* and potential phorophyte trees without *D. lindenii* during both flooded (2016) and not flooded periods (2018).

This apparent extreme fungal specificity for one OMF, *Ceratobasidium* Clade 2, is similar to that reported for mycoheterotrophic orchids (McKendrick et al., 2002; Selosse et al., 2002), terrestrial orchids (Thixton et al., 2020), and some epiphytic orchids (Otero et al., 2002, 2004; Graham and Dearnaley, 2012; Rammitsu et al., 2019, 2021a,b). Our findings of potential high specificity with *Ceratobasidium* Clade 2 aligns with previous studies demonstrating the importance of *Ceratobasidium* taxa supporting healthy populations of other epiphytic orchids. For instance, Qin et al. (2021) and Rammitsu et al. (2019) reported on other leafless epiphytic orchids that have a high specificity for single *Ceratobasidium* species. Furthermore, *Ceratobasidium* Clade 2 is conspecific (>99% similar) with *Ceratobasidium* (Dlin-394) that was isolated and brought into culture from roots of *D. lindenii* that was used to germinate *D. lindenii* seeds (Hoang et al., 2017).

We also observed evidence of possible specificity in some of the other co-occurring epiphytic orchids, but the sample size was small for many of these epiphytic orchids and clear hypotheses could not be tested. Nevertheless, these orchids associated with different *Ceratobasidium*. For example, *Ceratobasidium* OTU 19 and 22 were detected primarily in *D. porrectus*, another leafless epiphytic orchid. We hypothesize, with a caveat of small sample size, that mature roots of leafless epiphytic orchids are dominated by a single OMF unique to that species.

In addition to traditional OMF, we detected low read abundances of ECM fungi in the roots of the epiphytic orchids examined. This is in contrast to aerial roots of *V. planifolia* which were heavily colonized by ECM fungi (Johnson et al., 2021). *Vanilla planifolia* is a hemiepiphytic orchid and it is possible that the ECM fungi in the aerial roots are from systemic colonization emanating from the terrestrial roots. ECM fungi have been commonly reported from terrestrial orchids, but except for those detected by Johnson et al. (2021) an abundance of ECM fungi has not been reported colonizing arial/epiphytic orchid roots.

Foliar orchids exhibited lower read abundances relative to *D. lindenii* and other leafless epiphytic orchids (data not shown) was observed in our study. We hypothesize that the greater photosynthetic capacity of foliar orchids provided by their leaves reduces their dependence on OMF for supplemental fungal carbon.

Amplicon sequencing enabled us to document the presence of ECM fungi that are resistant to culturing in the orchid root communities. However, we were not successful in recovering species

of Tulasnellaceae. Whether this was actually due to very low abundance of these species is not clear. Some primer bias of the primer pair ITS86F/ITS4 for *Tulasnella* species has been reported and this primer pair is likely poor for detecting *Tulasnella* spp. (Tedersoo et al., 2015; Vogt-Schilb et al., 2020; Johnson et al., 2021; Rammitsu et al., 2021a). Thus, future work using primers that are not biased towards Tulasnellaceae is needed.

While bark is not a carbon source for orchids (Eskov et al., 2020), it is the likely source of the OMF that epiphytic orchids need for establishment including seed germination and seedling growth (Rasmussen et al., 2015). Pellitier et al. (2019) documented that tree bark can serve as an environmental filter for the fungal communities available to epiphytic orchids. Thus, the distribution of OMF in tree bark throughout an orchid's range could influence its fine-scale distribution. *Ceratobasidium* OTU Clade 2 was recovered from all trees with *D. lindenii*, the fungus was also recovered in low abundance from many potential phorophytes without the orchid, indicating that additional studies are necessary to comprehend the factors that contribute to the fine-scale distribution of *D. lindenii* beyond the presence of the required OMF. Although several *A. glabra* trees, the other phorophyte of *D. lindenii* in Florida, were sampled, we were not successful in obtaining sequences from those samples. Thus, attempts should be made to sample sufficient numbers of *A. glabra* to better understand the situation in Florida. Additionally, *D. lindenii* in Cuba is found on several phorophyte species in comparison to the two primary phorophyte species associated with *D. lindenii* in Florida. Therefore, a fuller understanding of factors influencing the fine-scale distribution of *D. lindenii* needs to include an analysis of Cuban phorophytes.

When present, *Ceratobasidium* Clade 2 in bark was recovered at low read abundances, i.e., <5% relative abundance even from bark samples collected adjacent to actively growing root tips of *D. lindenii*. If *Ceratobasidium* Clade 2 is functioning as a saprobe in bark, then it is likely an inefficient saprobe and being outcompeted by more efficient saprotrophic fungi in the bark fungal community.

Although some *F. caroliniana* in Site 4 had *Ceratobasidium* Clade 2 it may be below the threshold of abundance to facilitate establishment and support the growth of naturally occurring plants (McCormick et al., 2016). Understanding site differences in terms of the presence/abundance of *Ceratobasidium* Clade 2 and other factors influencing establishment is crucial to sustaining populations of *D. lindenii* and preventing 'senile' populations', an ageing orchid population that lacks seedling recruitment (Rasmussen et al., 2015).

The findings of this study indicate that *D. lindenii,* has high specificity for a specific taxon of *Ceratobasidium*, Clade 2. While this study provides data that suggest that the presence/absence (or very low abundance) of the required OMF influences which tree *D. lindenii* is likely to establish and persist, the fungus is probably not the sole factor driving fine-scale distribution. Future studies should focus on the role of abiotic factors, such as bark characteristics like pH and phenolics, on *Ceratobasidium* growth, as well as the ability of the orchid to establish on the tree surface. Pellitier et al. (2019) demonstrated that fungal communities are likely affected by pH and total phenolic content, therefore experiments to test this hypothesis should consider other abiotic factors.

This study establishes the usefulness of amplicon sequencing as a method to examine fungal communities in the roots of endangered orchids such as *D. lindenii*. Sampling the actively growing root tips provides a non-destructive sampling method for future studies of this and other threatened and endangered orchids. Naturally occurring *D. lindenii* appears to partner with a specific undescribed species of *Ceratobasidium*. However, lab-grown explants of *D. lindenii* have low abundance so long-term survival and successful reintroduction to natural habitats should account for potential phorophytes with abundant *Ceratobasidium* present for a viable conservation method. Additionally, while the presence of the required fungus is necessary for establishment of the orchid on a particular tree, it is likely that other factors which impact its fine-scale distribution, are also involved. Understanding how the difference in OMF abundance between naturally occurring plants and explants and the factors influencing successful establishment on phorophytes are needed to enhance the success of efforts to augment the population of the Ghost Orchid and refine conservation actions.

Data availability statement

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found at: NCBI; PRJNA948888.

Author contributions

LJ and MC contributed to the study conception, performed sample preparation, and data collection. LJ and GM wrote the first draft of the manuscript with an initial review by LZ. All authors contributed with comments on the later versions of the manuscript and approved of the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1057940/full#supplementary-material

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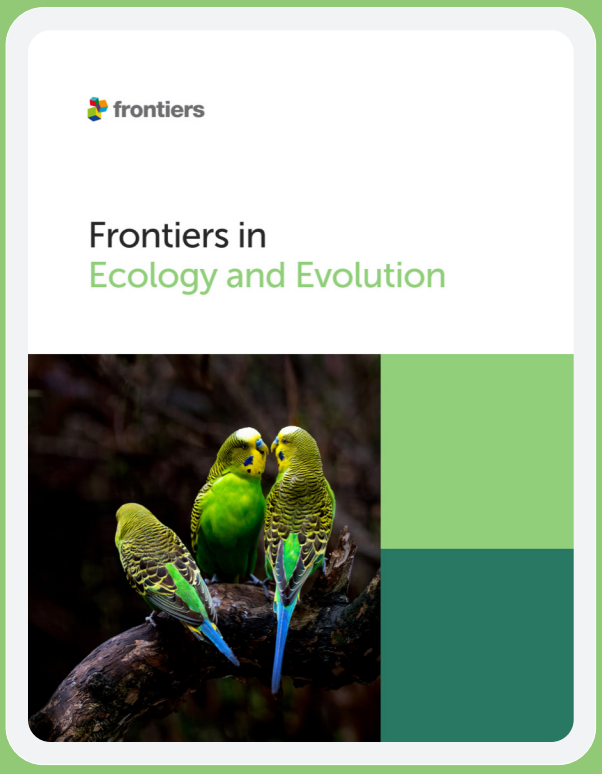

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