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Symbiont abundance can affect host plant population dynamics

--Manuscript Draft--

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Abstract:	Premise of the Study: Symbioses are almost universal, but little is known about how symbiont abundance affects host performance. Many orchids undergo vegetative dormancy and frequent and protracted dormancy have been associated with population declines. If mycorrhizal fungi affect host plant performance, those effects are likely to alter patterns of vegetative dormancy. The goal of this study was to determine whether the abundance of mycorrhizal fungi is related to the likelihood of entering dormancy and whether fungal abundance varied with dormancy duration in the federally listed threatened orchid, Isotria medeoloides. Methods: We studied three populations of the threatened North American terrestrial orchid, Isotria medeoloides, with long term emergence data and evaluated the relationship between the abundance of associated mycorrhizal fungi (Russulaceae) and orchid dormancy and emergence. Mycorrhizal fungi in soil adjacent to orchids were quantified in two ways. First, ectomycorrhizal (ECM) fungi on adjacent root tips were identified using DNA sequencing to determine their phylogenetic relationship to fungi that are known to form mycorrhizae with I. medeoloides. Second, we extracted DNA from soil samples and used quantitative real-time PCR to estimate the abundance of Russulaceae hyphae adjacent to each orchid. Key Results: We found that the abundance of Russulaceae, both in the soil and on nearby ECM root tips, was significantly related to orchid prior emergence. Both abundance and prior emergence history were predictive of future emergence. Conclusions: These results suggest that the abundance of mycorrhizal fungi can influence orchid population dynamics and is an essential component of orchid conservation.
Keywords:	Isotria medeoloides; orchid; Orchidaceae; dormancy; mycorrhizal fungi; Russula
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Corresponding Author's Name: Melissa McCormick
Date: 9/15/16

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[last revised 25 August 2015]

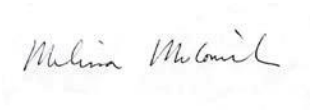
Smithsonian Environmental Research Center

8 December 2016
Pamela Diggle (Editor-in-Chief)
Department of Ecology and Evolutionary Biology
University of Connecticut
Storrs, CT 06269-3043, USA

Dear Dr. Diggle,

Please find attached the revised paper "Symbiont abundance can affect host plant population dynamics" for consideration for publication in the American Journal of Botany. We appreciate the thoughtful suggestions and efforts of the associate editor and have now revised this paper as suggested. Please feel free to contact me if there are questions that remain or issues we have inadequately dealt with. I have included our responses to reviewer comments below.

Sincerely,



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Responses to Editor's comments:

1. As per AJB's Instructions to Authors regarding Locality Information: Manuscripts that report data from individual populations must include locality information for each of the populations sampled if this information is not provided with an associated voucher specimen. Please be as precise as necessary for the site to be revisited by subsequent researchers. Coordinates as obtained from a GPS unit are ideal. A waiver of this requirement for locality information may be granted for rare, threatened, or endangered species.
***This requirement has been waived, as we worked with a Federally-listed Threatened species and releasing the location information is specifically prohibited.

2. Throughout the results, there are times when only p-values are presented for the statistics. Please also include the statistic value and df in all cases when reporting statistics in the results.
***Test statistics and df have now been added to all the places where only p-values were previously listed in the Results.

3. Table 2 is referred to twice on P19 (last two paragraphs of the results), yet there is no Table 2 included with the ms text.
***We have removed all references to Table 2, it appears to be a residual of a much earlier version.

4. Would you like to thank the reviewers in the acknowledgements? It is not required, but we recommend it, especially if you found their comments helpful. (They don't, as a rule, see your final response letter.)
***We now thank the reviewers and the editor for helpful comments on previous versions.

Smithsonian Environmental Research Center

4 December 2016
Pamela Diggle (Editor-in-Chief)
Department of Ecology and Evolutionary Biology
University of Connecticut
Storrs, CT 06269-3043, USA

Dear Dr. Diggle,

Please find attached the revised paper "Symbiont abundance can affect host plant population dynamics" for consideration for publication in the American Journal of Botany. We appreciate the thoughtful suggestions and efforts of the associate editor and have now revised this paper as suggested. Please feel free to contact me if there are questions that remain or issues we have inadequately dealt with. I have included our responses to reviewer comments below.

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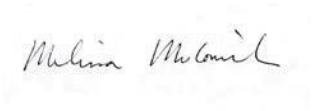

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Responses to Editor's comments:

I apologize for not catching this earlier, but was puzzled by the first sentences in the results (quoted below). The OTUs from ECM roots were a lot more diverse than the Isotria OTUs. Assuming that 'all' in line 289 refers to 'all ECM sequences' it doesn't make sense to say that the more diverse ECM fungi all fell into the smaller number of taxonomic clades represented by the less diverse Isotria sequences.
I have now changed the wording to "All DNA sequences from ECM roots obtained with the Russulaceae-specific primers fell within the Russulaceae, demonstrating that the primers targeted the desired taxa, and have been deposited in Genbank (Accessions KX528232-KX528327)"

How about switching the subject on line 289 from 'all ECM' to all fungi from the orchid roots, as in:-

The resulting alignment had 638 sites. On the phylogenetic tree, all fungi from I. medeoloides roots also belonged to diverse clades in Russula or Lactarius (Fig. 2), verifying that the
291 Russulaceae we quantified on root tips and in the soil targeted the desired fungi but also
292 indicating the breadth of fungi associating with I. medeoloides.

***Thank you for this suggestion. I agree that it is confusing as it was originally written and have changed to wording suggested (L289)

286 RESULTS
287 All DNA sequences from ECM roots obtained with the Russulaceae-specific primers
288 belonged to Russula or Lactarius and have been deposited in Genbank (Accessions KX528232-
289 KX528327). The resulting alignment had 638 sites. On the phylogenetic tree, all fell within
290 taxonomic groups containing fungi from I. medeoloides roots (Fig. 2), verifying that the
291 Russulaceae we quantified on root tips and in the soil targeted the desired fungi but also
292 indicating the breadth of fungi associating with I. medeoloides.

Editor Comments:
For figs 3, 5, 6, the figure legend is not correct. it specifies the identity of the line, but I think it should say what the gray vs. black circles represent. Also, do the lines have any biological meaning? If not, better to just present means and errors.

***Thank you for catching this. In figure3, 5, and 6, I have changed the wording to refer to grey and black symbols, rather than lines. I have removed the lines in figs 3, 4, 5, and 6.

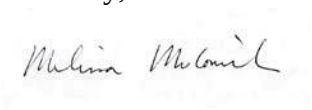
Smithsonian Environmental Research Center

24 November 2016
Pamela Diggle (Editor-in-Chief)
Department of Ecology and Evolutionary Biology
University of Connecticut
Storrs, CT 06269-3043, USA

Dear Dr. Diggle,

Please find attached the revised paper "Symbiont abundance can affect host plant population dynamics" for consideration for publication in the American Journal of Botany. In this paper, we examine how the abundance of symbionts can impact plant population dynamics. Specifically, we quantify how the abundance of mycorrhizal fungi can affect orchid dormancy. Recent studies have suggested that symbiont community composition can impact host physiology and population dynamics, but none have examined the effects of symbiont abundance. To truly understand how symbionts affect host distribution, it is critically important to consider symbiont abundance, in addition to composition. These results have implications for the vast majority of the earth's species that depend upon symbiotic associations, with particular relevance for the vast majority of plant species that depend on associations with mycorrhizal fungi. We appreciate the thoughtful suggestions and efforts of the associate editor and have now revised this paper according to their suggestions. Please feel free to contact me if there are questions that remain or issues we have inadequately dealt with. I have included our responses to reviewer comments below.

Sincerely,



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Associate editor comments. I am again recommending minor revision, mainly to ensure that the new Bayesian phylogenetic analysis is thorough and fully convincing. I appreciate that the authors took the reviewer suggestions seriously and look forward to a next and final version.

1. line 103, 123. Edited in abstract but not in intro. In the intro, the orchid remains 'federally threatened'. Do you want to leave the text as is?
***Thank you for catching this. It has now been changed in L103.
2. line 287, Clarify. Did the following apply to the number of sites in the alignment? If so, perhaps 'The resulting alignment had 638 sites' rather than 'The resulting tree had a sequence length of 638bp'
***This has been re-worded as suggested in L287.
3. To eliminate confusion based on the double meaning of rtPCR, perhaps add technical details. Rather than:
Line 249 'To quantify Russulaceae abundance in the soil, we conducted quantitative real-time PCR (qPCR).'
'To quantify abundance of Russulaceae DNA in the soil, we conducted quantitative real-time PCR (qPCR) of the ITS2 region.'
***Thank you for this suggestion. L149 has now been reworded as suggested.

4. Bayesian analysis and Fig. 2. While the Bayesian analysis that replaces the original UPGMA is an improvement, I have a couple of suggestions. Fig. 2 does offer convincing evidence that the mycorrhizal OTUs are somewhere near Russula. While the underlying message is not in doubt, the outgroups are very distant from ingroups Lactarius and Russula, and as a result, Russula appears paraphyletic and basal to Lactarius. This conflicts with most published phylogenies. A more careful phylogenetic analysis with closer outgroups should provide more support for nesting OTU12, OTU20 and OTU5 within Russula rather than as basal divergences of uncertain affiliation, as they now appear in Fig. 2. Russula KP348036, which appears with OTUs that are basal in the tree in Fig. 2 should rather appear in a clade with almost all other Russula species. (This is based on my BLAST search suggesting it is related to the iconic red-capped Russula emetica.)

Buyck et al. 2008 could get away with distant outgroups because they had available multiple, more highly conserved loci to use to construct their tree. More appropriate outgroups for ITS comparisons would be Boidinia aculeata, Boidinia furfuracea, or other similar species (Larsson and Larsson, Mycologia, 2003).
***The Bayesian analysis has now been re-run with Boidinia parva and Gloeocystidiellum rajchenbergii as outgroups. These fell within the most closely related genera identified in Larsson and Larsson, though we could not use the exact species that were in Larsson and Larsson because they sequenced a different region than we did, which would have greatly affected the resulting alignment. Indeed, this did produce a tree with better support for the OTUs.
5. (2) Provide some evidence that the final post burnin sampling represented a good sampling of the posterior distribution. Was the split frequency below 0.01 when the burnin period was finished? If not, more generations and a higher burnin proportion might reveal stronger support for some branches.
***L248-250: we have now added text indicating that the split frequency had declined to 0.008 after the burn-in period, suggesting that the burn-in used was adequate to provide a good sampling of the posterior distribution.

This said, it is unlikely that overall support for the phylogeny will be high. Using Bayesian phylogenetics, as the authors are doing, to show the relationships of the short OTU DNA sequences makes very good sense. It is understandable and consistent with other studies of the Russulaceae that the backbone relationships do not receive support.

Symbiont abundance can affect host plant population dynamics

Rachel Rock-Blake[2], Melissa K. McCormick[3,4], Hope E.A. Brooks[3], Cynthia S. Jones[2], Dennis F. Whigham[3]

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ABSTRACT

Premise of the Study: Symbioses are almost universal, but little is known about how symbiont abundance can affect host performance. Many orchids undergo vegetative dormancy and frequent and protracted dormancy have been associated with population declines. If mycorrhizal fungi affect host plant performance, those effects are likely to alter patterns of vegetative dormancy. The goal of this study was to determine whether the abundance of mycorrhizal fungi is related to the likelihood of entering dormancy and whether fungal abundance varied with dormancy duration in the federally listed threatened orchid, *Isotria medeoloides*.

Methods: We studied three populations of the threatened North American terrestrial orchid, *Isotria medeoloides*, with long term emergence data and evaluated the relationship between the abundance of associated mycorrhizal fungi (Russulaceae) and orchid dormancy and emergence. Mycorrhizal fungi in soil adjacent to orchids were quantified in two ways. First, ectomycorrhizal (ECM) fungi on adjacent root tips were identified using DNA sequencing to determine their phylogenetic relationship to fungi that are known to form mycorrhizae with *I. medeoloides*. Second, we extracted DNA from soil samples and used quantitative real-time PCR to estimate the abundance of Russulaceae hyphae adjacent to each orchid.

Key Results: We found that the abundance of Russulaceae, both in the soil and on nearby ECM root tips, was significantly related to orchid prior emergence. Both abundance and prior emergence history were predictive of future emergence.

Conclusions: These results suggest that the abundance of mycorrhizal fungi can influence orchid population dynamics and is an essential component of orchid conservation.

Keywords: (3-10) *Isotria medeoloides*; orchid; Orchidaceae; dormancy; mycorrhizal fungi; *Russula*

INTRODUCTION

Symbioses are essential for nearly all organisms, with mycorrhizal associations being an important symbiosis for the vast majority of terrestrial plants (Brundrett, 2009). The dynamics of the quantitative relationships between symbiotic partners can have important ecological and evolutionary consequences (Bruna et al., 2014), but only a few studies have quantified how the abundance of mutualist partners influences population dynamics (Lovelock and Miller, 2002; McCormick et al., 2009; McCormick et al., 2012; Vannette and Hunter, 2013).

The Orchidaceae is perhaps the largest family of flowering plants in the world (Dressler, 1993). Like most plants, orchids rely on associations with fungi but differ in that there is little evidence that the mycorrhizal interaction is mutualistic. Only one study has demonstrated definitively that the fungus may benefit from interactions with orchids (Cameron et al. 2008), though a few other studies have hypothesized that carbon transfer from orchids to mycorrhizal fungi explains depleted concentrations of ^{13}C in orchid hosts (e.g., Hynson et al., 2009; Liebel et al., 2015). It is widely assumed that the orchid is the only partner that benefits (Rasmussen 2002) and that benefit results from the digestion of the fungus by the orchid. The considerable resources that orchids gain from fungi is demonstrated by the presence of species that never emerge aboveground (Bougoure et al., 2009; 2010) and orchid genera that have lost their ability to obtain carbon through photosynthesis and are thus mycoheterotrophic (e.g., Barrett et al., 2010; Motomura et al., 2010; Liebel and Gebauer, 2011; Lee et al., 2015). In addition to obtaining resources from mycorrhizae, some orchids are known to be highly specific with regard to mycorrhizal symbionts that are required for seed germination (Yam and Arditti, 2009). Protocorms of all orchids require fungi for growth, and mycoheterotrophic species are fully dependent on fungi for growth and reproduction (Rasmussen, 2001; McCormick et al., 2012). In

addition, all orchids interact with fungi to varying degrees for growth beyond the seedling stage (Girlanda et al., 2011; Stöckel et al., 2014).

The importance of mycorrhizae throughout the life cycle of orchids is clear, but an intriguing characteristic of many orchid species is that they can undergo extended periods, called vegetative dormancy, during which they fail to produce any above-ground tissues during one or more growing seasons, but remain physiologically active (Shefferson et al., 2012). Such vegetative dormancy is often associated with plant stress and may be important for survival (Gremer et al., 2010; Gremer et al., 2012). However, for vegetative dormancy to be a successful strategy, plants must have higher survival or, at least not substantially less growth, than they would have not entering dormancy (Shefferson et al., 2014). What triggers orchids to enter or emerge from dormancy is unknown, but it may be related to plant nutritional status which, in turn, may be related to environmental stress (Gremer et al., 2010). Based on studies of fully mycoheterotrophic species (e.g., Bougoure et al., 2010), it can be assumed that during the time spent below-ground orchids remain physiologically active. It has therefore been proposed that orchids that are green and photosynthesize when they emerge may rely more heavily on their mycorrhizal fungi for nutrients, especially carbon, when they are vegetatively dormant (Gill, 1989; Shefferson et al., 2005).

The presence of appropriate mycorrhizal fungi is clearly essential for successful establishment and sustainability of orchid populations. What is less clear is whether or not the abundance of the appropriate mycorrhizal fungi is also important. The abundance of mycorrhizal fungi has been shown to support increased seed germination and protocorm development of three terrestrial orchids (McCormick et al., 2012), but whether fungal abundance is equally important for other orchid life history stages remains to be determined (McCormick and Jacquemyn, 2014).

When appropriate mycorrhizal fungi are absent, mortality results. Swarts and Dixon (2009) found that the loss of mycorrhizal fungi affected the health of orchid populations and after 1-2 years without the fungi being present, all plants died. Yet the extent to which mycorrhizal fungi contribute to the support of mature photosynthetic orchids, especially during periods of vegetative dormancy, is still an open question (Girlanda et al., 2011; Sommer et al., 2012; McCormick and Jacquemyn, 2014; Liebel et al., 2015).

Dormancy is thought to be a response to stress or to having insufficient nutrient stores to produce above ground shoots (Gremer et al., 2010) and the presence or abundance of appropriate mycorrhizal fungi may affect the likelihood of plants entering dormancy. Similarly, if dormant plants rely on mycorrhizal fungi for a large portion of their nutrition, survival and re-emergence are also likely to be affected by the presence and abundance of appropriate fungi (Shefferson et al., 2003; Shefferson, 2009). If symbiont abundance or identity affects orchid emergence or dormancy duration then they will also, by extension, affect rates of reproduction and patterns of outcrossing and gene flow such as has been demonstrated for a mycoheterotrophic non-orchid species (Logacheva et al., 2014).

In a previous study we found that *Isotria medeoloides*, a Federally listed threatened photosynthetic orchid that has frequent periods of vegetative dormancy, forms mycorrhizae with a variety of species in the Russulaceae (McCormick et al., 2013). Here we tested whether the abundance of mycorrhizal symbionts belonging to the Russulaceae was related to dormancy patterns. We quantified Russulaceae DNA in the soil and on ectomycorrhizal (ECM) tree roots adjacent to individual plants that were either emergent or dormant for different durations. We used a phylogenetic analysis to ensure that the fungi we quantified on ECM root tips and in nearby soil belonged to taxa that could likely serve as mycorrhizal associates of *I. medeoloides*.

We hypothesized that: 1) Russulaceae hyphae in the soil and the number of mycorrhizal root tips containing Russulaceae in soil samples adjacent to the orchids would be positively related to the presence of individuals that had emerged aboveground; 2) Time since last emergence would reflect the abundance of Russulaceae in the soil and; 3) Future emergence could be predicted by the abundance of Russulaceae hyphae in the soil and by the number of mycorrhizal root tips containing Russulaceae in soil samples adjacent to individual plants. To determine whether the model developed with data from hypotheses 1-3 could predict emergence in a new population, we sampled soil mycorrhizal fungi and monitored plant emergence in a separate study.

MATERIALS AND METHODS

Study species—*Isotria medeoloides* (Pursh) Raf. is a federally threatened orchid that occurs in forests in eastern North America (Mehrhoff, 1989). It emerges in the early spring and each plant produces a single compound leaf composed of a whorl of five leaflets (Mehrhoff, 1989; Fig 1a). Underground, each plant has a short stem with up to five short (up to 10cm long), thick (~0.25cm diam.) roots that are colonized by mycorrhizal fungi (Fig. 1b). Mycorrhizae in *I. medeoloides* involve fungi in the Russulaceae and two genera, *Russula* and *Lactarius*, have been identified by DNA sequencing of the ITS fungal barcoding region using fungus-specific PCR primers in root sections with visible pelotons (McCormick et al., 2012). No typical orchid mycorrhizal fungi in the genera *Tulasnella* or *Ceratobasidium* have ever been identified in *I. medeoloides* roots, nor have any fungi successfully been cultured. An ectomycorrhiza is a type of mycorrhiza in which the fungal associate forms a sheath around root tips before penetrating the root structure. All Russulaceae are obligately ECM, primarily with woody plants.

Isotria medeoloides populations are small, and the species has been in decline across its range and, thus, has been the focus of monitoring efforts (Mehrhoff, 1989; von Oettingen, 2008; Brumback et al., 2011; Cairns, 2012). Individual plants can persist underground in a state of vegetative dormancy for up to nine years (Cairns, 2001). During these periods of dormancy no part of the plant emerges above ground, but the plants remain physiologically active (Shefferson et al., 2012). Survival during these sustained dormancies is hypothesized to be the result of relationships with mycorrhizal fungi (McCormick et al., 2012). Whether a plant emerges in the spring is largely determined by whether or not it produced an overwintering bud during the previous growing season. Plants that produce a bud almost always emerge aboveground the

144 following spring, while those that do not form a bud remain dormant (Gregg 2011, McCormick
145 et al., 2015).

146 ***Study Sites*—**We conducted our studies at three sites. The first study was conducted from
147 2010-2013 at a site in the northern range of distribution of the species (Mount Teneriffe, New
148 Hamphsire - MTNH) and a site in the southern part of the range (Prince William Forest Park,
149 Virginia - PRWI). At the northern site we studied a population that consisted of an average of
150 113 emergent plants (Cairns, 2001) and was considered to be stable. The number of emergent
151 plants at the southern site was composed of multiple small subpopulations, averaging between 13
152 and 42 emergent plants that were in decline based on monitoring data (McCormick et al., 2015).

153 At each site the location of all plants was determined and all individuals were monitored
154 yearly. The Virginia populations were monitored by staff of the Smithsonian Environmental
155 Research Center (SERC) and the New Hampshire population was monitored by Sara Cairns of
156 the New Hampshire Natural Heritage Bureau.

157 In 2014, we conducted a second study at Fort A. P. Hill, Virginia (FAPH), located 70 km
158 south of the PRWI site. The FAPH site had multiple sites with small numbers of *I. medeoloides*,
159 all of which had been monitored for at least four years prior to this study (McCormick et al.
160 2015). The total population size at this site ranged from 27 to 58 emergent plants and was stable
161 or recovering, based on monitoring data (McCormick et al., 2015).

162 ***Sampling Methods*—**In order to test the hypothesis that the abundance of appropriate
163 mycorrhizal fungi contributed to the likelihood of emergence, we selected individual plants at
164 each location based on the number of years since they had last emerged. We selected a four-year
165 range because Cairns (2001) found that 95% of plants that re-emerged from dormancy did so
166 within five years. In study 1, we selected plants at PRWI and MTNH based on the number of

years that they had been dormant. Controls were locations where *I. medeoloides* had never been documented. The categories were: controls, plants that were emergent at the time of the study, plants that that been dormant for one year - last emerged in 2011, and plants that had been dormant for 3-4 years - last emerged prior to 2010. Ten locations were sampled for each of the four categories for PRWI. Eight locations with emergent plants and four locations for each of the other categories were sampled at MTNH. In study 2, plants at FAPH were also grouped into four categories, but because of the small population sizes allocation of numbers of samples to each category was somewhat unbalanced. The four categories were: control (no plants; 10 locations), plants that were emergent in 2014 (10 locations), plants that were dormant in 2014 but had emerged in 2013 (dormant for 1 year; 3 locations), and plants that last emerged prior to 2012 (dormant for 2-5 years; 3 locations).

One soil core (2.5 x 10 cm) was collected at a distance of 10 cm away in a random direction from the marked location of each individual plant. The distance was chosen to avoid damaging orchid roots, yet staying close enough to obtain a representative sample of associated ECM root tips of trees and shrubs. Samples were sieved (2 mm mesh screen) to remove rocks and collect root tips. Sieved samples that were collected as part of the first study were rinsed in the sieve to remove any remaining soil. In both studies, soil that passed through the sieve was collected for later DNA analysis of Russulaceae abundance.

In study 1, ECM root tips were submerged in water, sorted under a dissecting microscope into morphotypes, and counted. Morphotypes were distinguished and grouped by color, shape, size, and surface hyphae extensions (see Brundrett et al. 1996 for method). Two root tips of each morphotype in each sample were selected for sequencing of ECM fungal DNA (see details

189 below). Root tips were sliced into two 0.5 mm diameter discs using a sterile scalpel. Each disc
190 was placed in a separate PCR tube with 9.5 μL of water.

191 Although Russulaceae are considered obligate ECM, patterns of root tip abundance do
192 not necessarily reflect the abundance of hyphae in the soil (Kjøller, 2006). Hyphae may have
193 relatively high abundances in soils with lower nutrient availability because the fungi must forage
194 over a larger volume to access required nutrients. Because orchid mycorrhizal fungi are not
195 thought to be actively attracted to orchid seeds and plants (Rasmussen, 1995), the abundance of
196 hyphae in the soil may be an important gauge for determining how frequently the fungi
197 encounter and colonize orchids. The dry sieved soils were freeze-dried and ground using a
198 mortar and pestle. DNA was extracted from 0.25 g of each soil sample using Powersoil® Soil
199 DNA Isolation kits (MO BIO Laboratories, Inc. Carlsbad, CA, USA).

200 In study 2, our focus was on the abundance of Russulaceae in the soil and the relationship
201 between fungal abundance and the presence of plants aboveground, as well as the abundance of
202 fungi in the soil when plants have not appeared aboveground for one or more years.

203 ***Polymerase Chain Reaction (PCR)—***To identify root morphotypes associated with
204 Russulaceae, we used direct PCR amplification (McCormick et al. 2009) on root disks, using
205 primers specific to the nuclear ribosomal internal transcribed spacer (ITS) of fungi in the
206 Russulaceae.

207 We used 25 μL PCR reactions containing 1.25 μL of each primer, 0.1 μL of bovine serum
208 albumin (BSA), and 12.5 μL of PCR Master Mix (2.0X RED Master Mix kit, Genesee Scientific,
209 San Diego, California, USA) on one disk from each root tip sampled. The root disk occupied
210 approximately 0.5 μL and the remaining 9.4 μL was sterile water. One section of each analyzed
211 root tip was amplified with the Russulaceae specific primer pair ITS3-R1A

(CATTCCGAGGGGCACACCCG, D. Lee Taylor, unpublished)/ITS4 (White et al., 1990), as follows: 1 min initial denaturation at 96°C, followed by 34 cycles of 30 seconds at 94°C, 30 seconds at 54°C, 1 min at 72°C. This was followed by 10 min at 72°C.

The second disk for each root tip was analyzed by amplification with a plant specific primer pair ITS1-P (TTATCATTTAGAGGAAGGAG, developed by T. D. Bruns)/ ITS4 (White et al., 1990). The PCR cycle for amplification of plant DNA was as follows: 2 min initial denaturation at 94°C, followed by 34 cycles of 30 seconds at 94°C, 30 seconds at 50°C, 1 min at 72°C. This was followed by 10 min at 72°C. This technique allowed us to identify the tree species that had formed ECM with Russulaceae. However, plant sequences amplified poorly and are not reported here. If the fungi for both of the root tip disks sampled from a morphotype were identified as Russulaceae we assumed that all roots with that morphotype were colonized by Russulaceae. In 28% of the morphotypes, only one of the two root tips disks amplified successfully. In these cases, sequences from identical-appearing morphotypes in other samples were used to determine whether all root tips could reasonably be counted as Russulaceae. If identification was still unclear, as was the case for two morphotypes, we selected additional root tips from the morphotype to analyze. This method allowed us to quantify support for Russulaceae fungi in the vicinity of each analyzed orchid and also the number of total ECM root tips that were occupied by Russulaceae.

To verify that Russulaceae fungi colonizing ECM root tips corresponded to potential mycorrhizal fungi for *I. medeoloides,* we sequenced the PCR product from each ECM root sample with a clear gel band when amplified with Russulaceae primers. PCR product was cleaned using ExoSap-IT (Affymetrix, Santa Clara, CA, USA), and approximately 20ng was

sequenced using BigDye v3.0 (ABI) as per manufacturer's instructions, cleaned using Sephadex G10-fine (GE Health Sciences), and run on an ABI 3130 sequencer.

***Phylogenetic Analysis*—**We constructed a phylogenetic tree of Russulaceae ECM sequences, the closest matching sequences in GenBank, and sequences from Russulaceae within the roots of nine *I. medeoloides* plants (McCormick et al., 2013). The tree was rooted in two outgroup taxa that belonged to genera identified by Larsson & Larsson (2003) in their phylogenetic analysis of Russulaceae. We first combined the ECM fungi into OTUs using 97% sequence similarity cutoff in Geneious (v. 8.1, Biomatters, Ltd.). We then aligned all OTUs using MAFFT alignment, implemented in Geneious, with auto algorithm, a gap open penalty of 1.53, and an offset value of 0.123. Selected sequences from GenBank and from *I. medeoloides* roots were also included and the final alignment was adjusted manually, resulting in a final alignment of 638bp. Relationships between taxa were visualized using a phylogeny constructed using MrBayes (v. 3.2.6; Huelsenbeck & Ronquist 2001) with HKY85 as the genetic distance model and gamma rate variation using a burn-in of 100,000 trees, a chain length of 1,100,000, 4 heated chains, and a heated chain temp of 0.2. After the burn-in period, the split frequency had declined to 0.008, suggesting this burn-in duration was sufficient to achieve adequate sampling of the posterior distribution. Trees were subsampled every 200 trees with a random seed.

Real-time Polymerase Chain Reaction (qPCR)— To quantify Russulaceae abundance in the soil, we conducted quantitative real-time PCR (qPCR) of the ITS2 region. We carried out 25 µL reactions containing 12.5 µL iQ SYBR Green PCR Super Mix (BioRad Laboratories, Hercules, CA), 20 ng DNA template in 8µl H_2O, and 1.25µL (10 mM) each of primers ITS3-R1A and ITS4 on an MJ Research Opticon DNA Engine with Continuous Fluorescence Detection (MJ Research, now Bio-Rad Laboratories, Hercules, CA, USA), as follows: initial

denaturation at 95°C for 5 min followed by 41 cycles of 15 s denaturation at 94°C, 30 s annealing at 54°C, and 30 s elongation at 72°C. Each sample was amplified in triplicate, and quantified using a standard curve. Four serial dilutions of genomic DNA from a pure culture of a *Russula* sp. isolated from a fruiting body were used to construct a standard curve (range: 0.001-1 ng target genomic DNA). In addition, a melting curve analysis was performed after each analysis to confirm the specificity of the qPCR.

***Statistical analyses*—** All statistical analyses were performed in R (3.2.2) using the R Stats package. The quantities of Russulaceae DNA found in the soil samples were natural log transformed to improve distribution normality. The number of root tips colonized by Russulaceae was counted and z-scored and centered prior to statistical analysis. In both study 1 and study 2, binary logistic regression models were used to test whether current and future emergence were significantly related to the abundance of Russulaceae fungi on ECM root tips and in the soil. The likelihood-ratio criterion was used in all analyses as a conservative test statistic. For all logistic regressions, Nagelkerke's R^2 was used as the coefficient of determination. Nagelkerke's R^2 was chosen due to its ability to provide an improvement over Cox and Snell's R^2. When appropriate, multicollinearity was tested through generalized variance inflation factors and tolerance (Myers, 1990; Menard, 1995).

In study 1, the independent predictor variables were ordered as follows: abundance of Russulaceae hyphae in the soil, number of root tips containing Russulaceae, and collection site. Re-ordering of the independent variables did not alter their significance or predictive contributions to the models. In both study 1 and study 2, the number of years since last emergence was used as a covariate. This measure of prior emergence was transformed and centered; a higher more positive number indicated most recent emergence.

280 We used two ANOVAs with ‘years since emergence’ and ‘site’ as independent variables
281 and ‘number of Russulaceae-colonized ECM root tips’ and the ‘abundance (ng of DNA) of
282 Russulaceae in the soil’ as dependent variables to determine the extent to which the abundance
283 of Russulaceae fungi could be related to time since last emergence. Abundance data were natural
284 log transformed prior to analysis to improve normality.
285

286 RESULTS

287 All DNA sequences from ECM roots obtained with the Russulaceae-specific primers
288 belonged to the genera *Russula* or *Lactarius* and have been deposited in Genbank (Accessions
289 KX528232-KX528327). The resulting alignment had 638 sites. On the phylogenetic tree, all
290 fungi from *I. medeoloides* roots also belonged to diverse clades in *Russula* or *Lactarius* (Fig. 2),
291 verifying that the Russulaceae we quantified on root tips and in the soil targeted the desired
292 fungi, but also indicating the breadth of fungi associating with *I. medeoloides*.

293 **Study 1:** As predicted, we found that the abundance of Russulaceae in the soil and in
294 adjacent ECM root tips were both significantly related to current orchid emergence in 2012, X^2
295 (7) = 24.03, $P < 0.001$ (see Table 1, Fig. 3). Nagelkerke's R^2 of 0.317 indicated a moderately
296 strong relationship. The likelihood-ratio criterion demonstrated that the Russulaceae in the soil
297 and on the adjacent root tips both made significant contributions to the overall model. For each
298 proportional increase in amount (i.e., nanograms) of Russulaceae DNA in the soil, the odds of
299 emergence increased by a factor of 1.12 (X^2 (1) = 10.54, $P < 0.001$), after controlling for all
300 other factors in the model. For each increase in the number of adjacent root tips colonized by
301 Russulaceae, the odds of orchid emergence increased by a factor of 2.32 (X^2 (1) = 6.37, P =
302 0.011). There was no significant effect of collection site (X^2 (1) = 0.21, P = 0.644) (MTNH vs.
303 PRWI). Multicollinearity did not impact the overall model and associated statistics, as indicated
304 by tests of generalized variance inflation factors and tolerance (Myers, 1990; Menard, 1995).

305 The abundance (ng) of Russulaceae DNA in the soil differed significantly among sites (F =
306 23.43, df = 1, $P < 0.001$) and among locations where plants had remained dormant for different
307 numbers of years (F = 20.01, df = 1, $P < 0.001$), and this pattern was similar across sites (F =
308 0.90, df = 3, P=0.554; Fig. 4).

Results also supported our third hypothesis. Because the model successfully predicted future 2013 emergence, X^2 (7) = 46.82, $P < 0.0001$ (see Fig. 5a). Nagelkerke's R^2 of 0.731 indicated a moderately-strong relationship. The likelihood-ratio criterion demonstrated that the number of adjacent root tips containing Russulaceae made a significant contribution the overall model. For each unit increase in the number of adjacent root tips colonized by Russulaceae, the odds of future orchid emergence increased by a factor of 38.79 (X^2 (1) = 14.02, $P < 0.0001$).

While there was no significant main effect of Russulaceae abundance in the soil, there was a significant interaction between Russulaceae abundance in the soil and the number of root tips. For each increase in the number of adjacent root tips colonized by Russulaceae in combination with each increase in ng of Russulaceae DNA in adjacent soil samples, the odds of orchid emergence increased by a factor of 1.48 (X^2 (1) = 6.21, $P < 0.01$). In short, emergence was more likely to occur when Russulaceae was abundant both on adjacent root tips and in the soil surrounding the orchid. In addition, there was a significant interaction between the abundance of Russulaceae in the soil and the collection site (X^2 (1) = 7.49, $P < 0.01$) (see Fig. 5B), and a significant interaction bet ween the number of root tips containing Russulaceae and the collection site (X^2 (1) = 6.03, $P < 0.01$) (see Fig 5C). This was because MTNH and PRWI had very different abundances of Russulaceae in the soil. To better interpret this interaction, an analysis was run separately for each collection site (i.e., Prince William and Mt. Teneriffe). In the follow up analyses, the interaction term between collection site and measures of Russulaceae (i.e., root tips and soil) was removed. This is because we were analyzing the effect of collection site on future emergence at the Prince William collection site separately from the Mt. Teneriffe collection site.

331 The model of the Prince William collection site successfully predicted future 2013
332 emergence, X^2 (3) = 18.30, $P < 0.0001$. Nagelkerke's R^2 of 0.626 indicated a moderately-strong
333 relationship. At the Prince William collection site there was a trending main effect of
334 Russulaceae in soil and a significant main effect of Russulaceae in root tips. For each
335 proportional increase in the amount of Russulaceae DNA in adjacent soil samples, orchid
336 emergence at PRWI increased by an odds ratio of 1.52 (X^2 (1) = 3.18, $P = 0.07$). In other words,
337 as Russulaceae in the soil increased, orchids at the PRWI collection site were more likely to
338 emerge. For each unit increase in the number of adjacent root tips colonized by Russulaceae,
339 orchid emergence at PRWI increased by an odds ratio of 62.05 (X^2 (1) = 15.70, $P < 0.001$). This
340 result demonstrated that as the number of adjacent root tips colonized by Russulaceae increased,
341 orchids at the PRWI collection site were more likely to emerge.
342 The model of the Mt. Teneriffe collection site successfully predicted future 2013
343 emergence, X^2 (3) = 9.62, $P < 0.05$. Nagelkerke's R^2 of 0.518 indicated a moderate relationship.
344 At the Mt. Teneriffe collection site there was no main effect of Russulaceae in soil (X^2 (1) =
345 0.001, $P = 0.97$) and a trending main effect of Russulaceae in root tips (X^2 (1) = 3.28, $P = 0.07$);
346 however, there was a significant interaction of root tips and soil. While neither measure of
347 Russulaceae in isolation was able to predict future emergence, together they strongly predict
348 future orchid emergence. For each proportional increase in the amount of Russulaceae measured
349 in both adjacent soil samples and root tips, orchid emergence at Mt. Teneriffe increased by an
350 odds ratio of 1.49 (X^2 (1) = 6.19, $P < 0.01$). In other words, as measures of Russulaceae in the
351 soil and root tips increased, orchids at the Mt. Teneriffe collection site were more likely to
352 emerge.
353

Study 2: As we predicted in our third hypothesis, we found that the model we developed for PRWI and MTNH also demonstrated a similar relationship between the abundance of Russulaceae in the soil and current orchid emergence in 2014 at Fort A.P. Hill, X^2 (1) = 5.411, P = 0.02, Nagelkerke's R^2 = 0.07 (see Fig. 6). However, it is worth noting that effect size is limited by the amount of variance available. In other words, this collection site contained homogeneous data. For each proportional increase in ng of Russulaceae DNA in the soil, the odds of emergence increased by a factor of 1.13, after controlling for all other factors in the model.

Finally, we found that the model that we developed, when used with data from FAPH, performed similarly in predicting 2015 future orchid emergence, X^2 (1) = 5.635, P = 0.018, Nagelkerke's R^2 of 0.067 (see Fig. 6). Again, the effect size was limited by the amount of variance. For each increase in the amount of Russulaceae DNA in the soil, the odds of future emergence increased by a factor of 1.14, after controlling for all other factors in the model. Tests of generalized variance inflation factors and tolerance (Myers, 1990; Menard, 1995) indicated that multicollinearity had a nonsignificant impact on the overall model and associated statistics.

DISCUSSION

While many papers have demonstrated the effect of symbiont presence on plant performance, the effect of symbiont abundance has been less well appreciated (e.g., Vannette and Hunter 2013). In this study we found a direct relationship between the abundance of mycorrhizal fungi and emergence from dormancy. Because the frequency of dormancy and the length of time that it persists have cascading effects on orchid population ecology (e.g., growth, reproduction, outcrossing), these results demonstrate that the abundance of mycorrhizal fungi can impact orchid population dynamics. These results also suggest that understanding the positive association between orchids and their mycorrhizal fungi is important for sustaining and increasing orchid populations and that patterns of orchid dormancy may reflect the abundance of mycorrhizal fungi near individual orchids.

We found that the abundance of Russulaceae DNA in soil samples and their colonization of adjacent mycorrhizal root tips were both significant predictors of orchid emergence. However, the patterns of the two measures differed among sites. In particular, both the numbers of ectomycorrhizal root tips and the concentration of Russulaceae DNA obtained from the soil differed between study sites. Within each site, locations with more Russulaceae DNA in the soil and more colonized ECM root tips were more likely to have *I. medeoloides* plants that were emergent rather than dormant.

Colonization of ECM root tips may reflect the degree of non-orchid plant support for the Russulaceae fungi associated with *I. medeoloides*, such that fungi that colonized many tree root tips might have access to more extensive carbon resources than those colonizing fewer roots, making them better able to support *I. medeoloides* growth. However, tree species and the light

393 and soil conditions experienced by individual trees all contribute to the carbon benefit obtained
394 by mycorrhizal fungi (e.g., Aguilar-Chama & Guevara 2016). These factors differ more among
395 sites, such as from New Hampshire to Virginia, than within sites, perhaps contributing to
396 differences among sites in the number of ECM root tips, while retaining the pattern of greater
397 Russulaceae root colonization being associated with greater probability of emergence within
398 sites.

399 Sampling soil at only one location for each orchid in the highly heterogeneous soil
400 environment may have introduced considerable noise into the assessment of mycorrhizal fungi
401 available to support *I. medeoloides.* Furthermore, differences in soil types among sites may have
402 created different extraction efficiencies that contributed to very different measured abundance of
403 Russulaceae among sites. Soils from PRWI and MTNH were very different, with the MTNH
404 soils being much richer in particulate organic matter than those at PRWI. Because of this, the
405 Russulaceae fungi in organic-rich soils might have been less well lysed during the DNA
406 extraction process. Soils from FAPH were very similar to those at PRWI and had similar
407 concentrations of Russulaceae DNA. Alternatively, these site differences may reflect differences
408 in how these fungi, which the DNA sequencing indicated included different species in the two
409 sites, distribute their growth (i.e., to root tip colonization vs. extramatrical hyphae) in different
410 environments. Despite these differences among sites, a significant positive relationship between
411 the abundance of Russulaceae in the soil and probability of orchid emergence persisted when
412 root tip colonization data were not available for hypotheses 3 and 4.

413 High variability in abundance of Russulaceae DNA was seen at locations where individuals
414 had last emerged prior to 2010. We speculate that locations with low abundances of Russulaceae
415 might be locations where *I. medeoloides* have died, while locations with abundant Russulaceae

might be locations with healthy plants that are preparing to re-emerge. However, because no portion of *I. medeoloides* is visible above ground when it is vegetatively dormant and we were reluctant to possibly damage the plant or its fungi by excavating around the plant location, the actual status of individual plants was not known unless they were emergent.

Increasing Russulaceae, whether in adjacent root tips or as DNA in the soil, had a greater effect at PRWI than at MTNH, particularly in the adjacent root tips. This may suggest that Russulaceae fungi may be related to dormancy and that extended dormancy may be associated with insufficient Russulaceae. However, at the Mt. Teneriffe site the future emergence of *I. medeoloides* was predicted by the interaction of Russulaceae abundance in the soil and on root tips. An intriguing possibility is that the orchid's future emergence is related to a different balance of Russulaceae distribution or to fungal species that differ in how biomass is allocated to colonization of root tips and soil volume. Furthermore, there was no significant main effect of collection site ($P = 0.18$; Mt. Teneriffe, NH, and Prince William Forest Park, VA), indicating that, regardless of collection site, increased Russulaceae in the soil and on adjacent root tips increased the likelihood of *I. medeoloides* emergence. The actual degree to which this occurred may, however, be a result of overall low emergence at PRWI versus MTNH. Future studies should test the relationship between Russulaceae abundance in the soil and on ECM root tips explicitly by using larger samples at a range of more heterogeneous collection sites.

All fungi sequenced on ECM root tips that scored positive for Russulaceae based on the presence of PCR product belonged to the genera *Russula* and *Lactarius*, the dominant genera in Russulaceae. Both genera have been found in mycorrhizal associations with *I. medeoloides* (Fig.2), so analysis was performed on the data using the Russulaceae family as a whole. However, it is worth noting that not all fungi in these genera are likely to be appropriate

mycorrhizal associates of *I. medeoloides*. Currently, only nine fungi have been identified from *I. medeoloides* roots and, although these fungi include examples spread across the Russulaceae, it is unknown what distinguishes the taxa that successfully form mycorrhizae with *I. medeoloides* or whether there are significant groups within Russulaceae that would not form functional mycorrhizae. In particular, some clades of *Russula* and *Lactarius*, especially the clade containing *R. xerampelina, R. flavisiccans, R. puellaris, R. abietina,* and *R. velenovskyi*, seemed to be overrepresented in *I. medeoloides* (6/9), compared to their lower dominance on ectomycorrhizal roots in adjacent root tips (86/214; Fig. 2). At a coarse taxonomic scale, *I. medeoloides* associated with *Russula*, as compared to *Lactarius* taxa, in approximately the same proportions as they were found on ECM root tips, perhaps suggesting less specificity than might be expected. By including all Russulaceae within our analysis, we have almost certainly overestimated the abundance of potential mycorrhizal associates. However, because the fungi identified in association with *I. medeoloides* did not form a distinct subclade, it was not possible to design PCR primers that were specific to only those fungi. Furthermore, since the fungi identified to this point in association with *I. medeoloides* have come from a small number of plants, they also likely do not represent the full extent of potential host fungi. To the extent that the fungi that form mycorrhizae with *I. medeoloides* track abundance patterns of the family, the family-level abundances will represent mycorrhizal abundance. It is possible that we could measure abundant Russulaceae DNA, yet have no *I. medeoloides* mycorrhizal fungi, but if the target mycorrhizal fungi were abundant we would measure abundant Russulaceae DNA. Using DNA from the whole family represents the best option available at this point and is a necessary starting point for examining relationships between mycorrhizal fungus abundance and *I. medeoloides* dormancy patterns.

462 Vegetative dormancy can make it challenging to understand the factors that contribute to the
463 decline of a rare or threatened species. Many orchids undergo dormancy, often as an adaptation
464 to stress or decline (Shefferson, 2009; Shefferson et al., 2012). For *Isotria medeoloides*, a
465 perennial terrestrial orchid growing in relatively low-light environments, the risk of emergence
466 (i.e., herbivory, trampling) may outweigh the benefits of photosynthesis and sexual reproduction
467 in a given year. Our results show that dormancy was associated with low abundances of
468 symbiotic mycorrhizal fungi in the soil. This suggested that a support network of beneficial fungi
469 was directly associated with emergence in this orchid species. While other factors such as light
470 availability can also contribute to orchids entering dormancy (Shefferson et al., 2012), our work
471 suggests that assessment of the abundance of host mycorrhizal fungi in the soil might serve as an
472 efficient diagnostic method for population health in lieu of long-term demographic studies. An
473 evaluation of the suitability of potential habitats for orchid reintroduction may also be possible
474 based on sampling soil for appropriate mycorrhizal fungi.

475 Because orchids cannot germinate or live without specific mycorrhizal fungi in natural
476 environments, they may be some of the first plants affected by changes in their mycorrhizal
477 symbionts (Bellgard and Williams, 2011). In this study, we found that orchid dormancy, and
478 hence population dynamics, was related not just to the presence, but also to the *abundance* of
479 mycorrhizal symbionts. Dormancy may be one of the first places that the effect of symbiont
480 abundance on individual performance can be detected, and at least 52 plant species in 10 plant
481 families engage in some sort of vegetative dormancy (Shefferson et al., 2012). Population
482 dynamics of these other plants could be similarly affected by factors that affect their mycorrhizal
483 fungi. However, even plants that do not enter dormancy may be affected by the abundance of
484 their mycorrhizal fungi. Over 80% of land plants associate with mycorrhizal fungi (Brundrett

2009) and the abundance of those fungi may affect a wide range of plant traits. For example, Vannette and Hunter (2013) found that the abundance of mycorrhizal fungi affected plant nutrient concentration and defensive structures, which altered their interactions with herbivores. The widespread importance of mycorrhizal associations for terrestrial plants suggests that these effects are likely much more widespread and that plant reproduction, community interactions, and population dynamics may be affected by the abundance of symbionts.

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Table 1: Abundance of Russulaceae in the Soil and on Root tips sampled from adjacent to plants that were emergent vs not emergent across all three sites. Values for Soil are ln (ng Russulaceae DNA/g dry soil). Values for Root tips are the number of ectomycorrhizal root tips colonized by fungi belonging to the Russulaceae.

		Prince William Forest Park, VA mean ± SE		**Mount Teneriffe, NH mean ± SE**		**Fort AP Hill, VA mean ± SE**
		Soil	Root tips	Soil	Root tips	Soil
Current	Emergent	-2.92 ± 1.06	153.4 ± 48.51	-6.43 ± 1.27	531.5 ± 100.63	-5.51 ± 1.16
	Not Emergent	-5.67 ± 0.77	116.63 ± 14.06	-4.51 ± 1.77	498 ± 66.76	-7.89 ± 1.22
Future	Emergent	-2.49 ± 0.61	249.75 ± 107.58	-5.3 ± 2.09	559.33 ± 114.28	-4.89 ± 1.62
	Not Emergent	-5.26 ± 0.72	112.06 ± 12.17	-4.01 ± 1.84	470.1 ± 77.67	-7.67 ± 1.01

Notes: Root tip values are raw values, and Soil values are natural log transformed.

608 Figure legends:

609 Figure 1: *Isotria medeoloides* a) aboveground and b) belowground. The flowering, emergent,
610 plant (a) consists of a single leaf, usually with five leaflets. Belowground (b), the plant base
611 (single arrow) includes a green bud for the next year's growth and bud scars from the previous
612 year's sprouts and a few relatively short (1-10cm), coarse roots (double arrow).

613 Figure 2: Bayesian inference phylogenetic tree showing the relationship between Russulaceae
614 fungi sequenced from ectomycorrhizal root tips adjacent to *I. medeoloides* plants and those
615 sequenced directly from *I. medeoloides* roots and their closest matches from GenBank.
616 Sequences from individual ectomycorrhizal root tips are designated 'Iso', followed by the site
617 (MTN for MTNH or PW for PRWI) and the sample number. OTUs indicate identical sequences
618 that were obtained from multiple ectomycorrhizal root tips. Each OTU is given a number and
619 that is followed by the number of root tips represented by that OTU in parentheses and a symbol
620 indicating the site where it was found (solid black downwards triangle for PRWI or open
621 upwards triangle for MTNH). Sequences from orchid roots are designated '*Isotria*', followed by
622 an identifier for the individual plant and population location. Numbers on the branches indicate
623 posterior probabilities for branches with probabilities greater than 0.70.

624 Figure 3: The amount of Russulaceae (z-scored) in the soil (black symbols) and in adjacent
625 ectomycorrhizal root tips (gray symbols). Comparison of plants that emerged in 2012
626 (Emergence) with plants that did not emerged (No Emergence).

627 Figure 4: The abundance of Russulaceae in the soil by years since an *I. medeoloides* last emerged
628 at a) Mount Teneriffe, NH, b) Prince William Forest Park, VA, and c) Fort A.P. Hill, VA. Note
629 the different scales on the axes.

Figure 5: The amount of Russulaceae (z-scored) and future emergence (2013) of the orchid *Isotria medeoloides* in Study 1 based on (a) measures of Russulaceae: soil (black symbols) or adjacent mycorrhizal root tips (gray symbols), (b) number of adjacent mycorrhizal root tips colonized by Russulaceae fungi by collection site Mount Teneriffe, NH (MTNH, black symbols) and Prince William Forest Park, VA (PRWI, gray symbols), and (c) Russulaceae in the soil by collection site Mount Teneriffe, NH (MTNH, black symbols) and Prince William Forest Park, VA (PRWI, gray symbols).

Figure 6: The amount of Russulaceae in the soil and emergence of the orchid *Isotria medeoloides* in Study 2 at Fort A. P. Hill, VA (FAPH) by current year of emergence (2014; black symbols) and future year of emergence (2015; gray symbols).

Figure1

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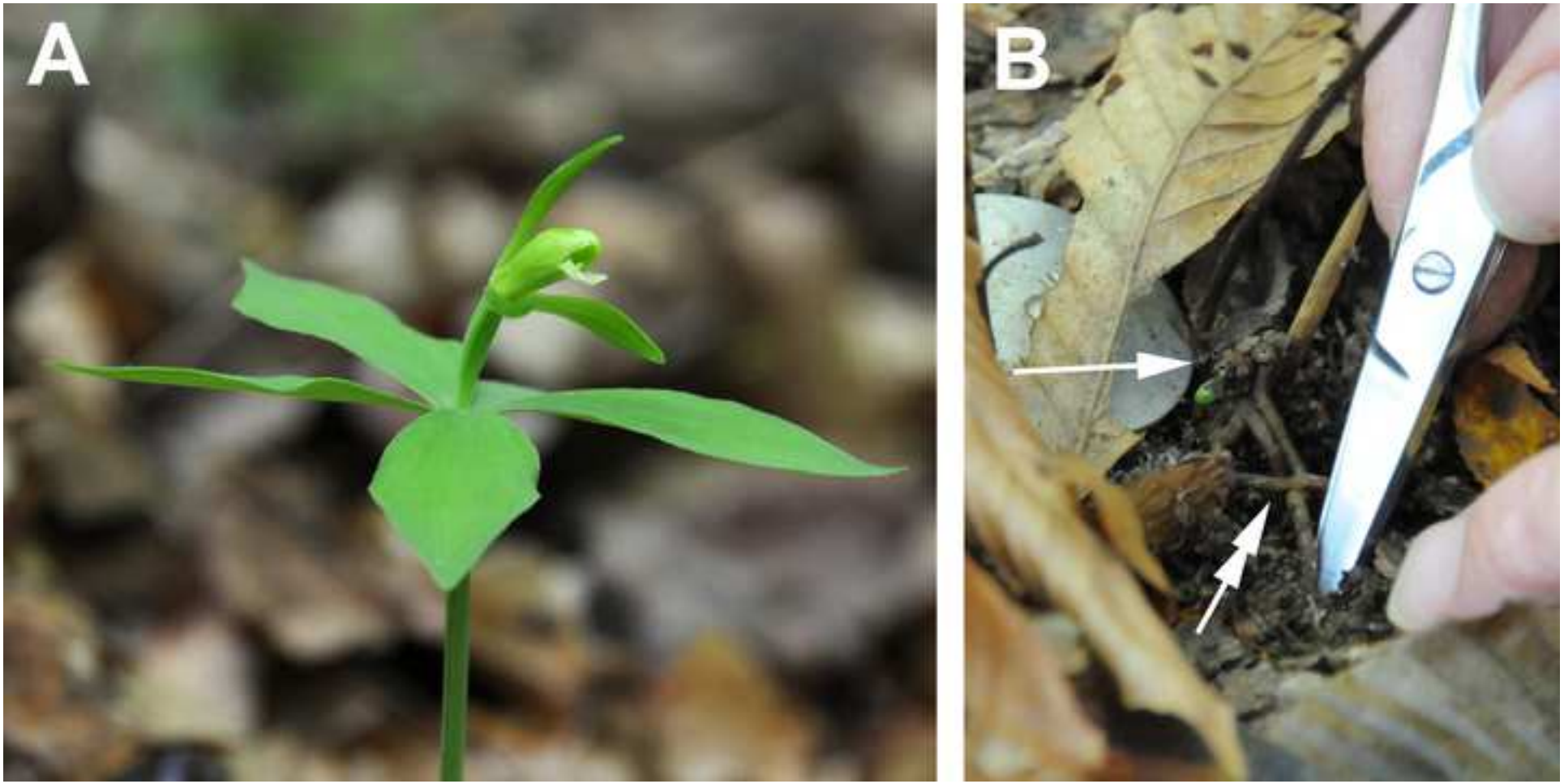

Figure2

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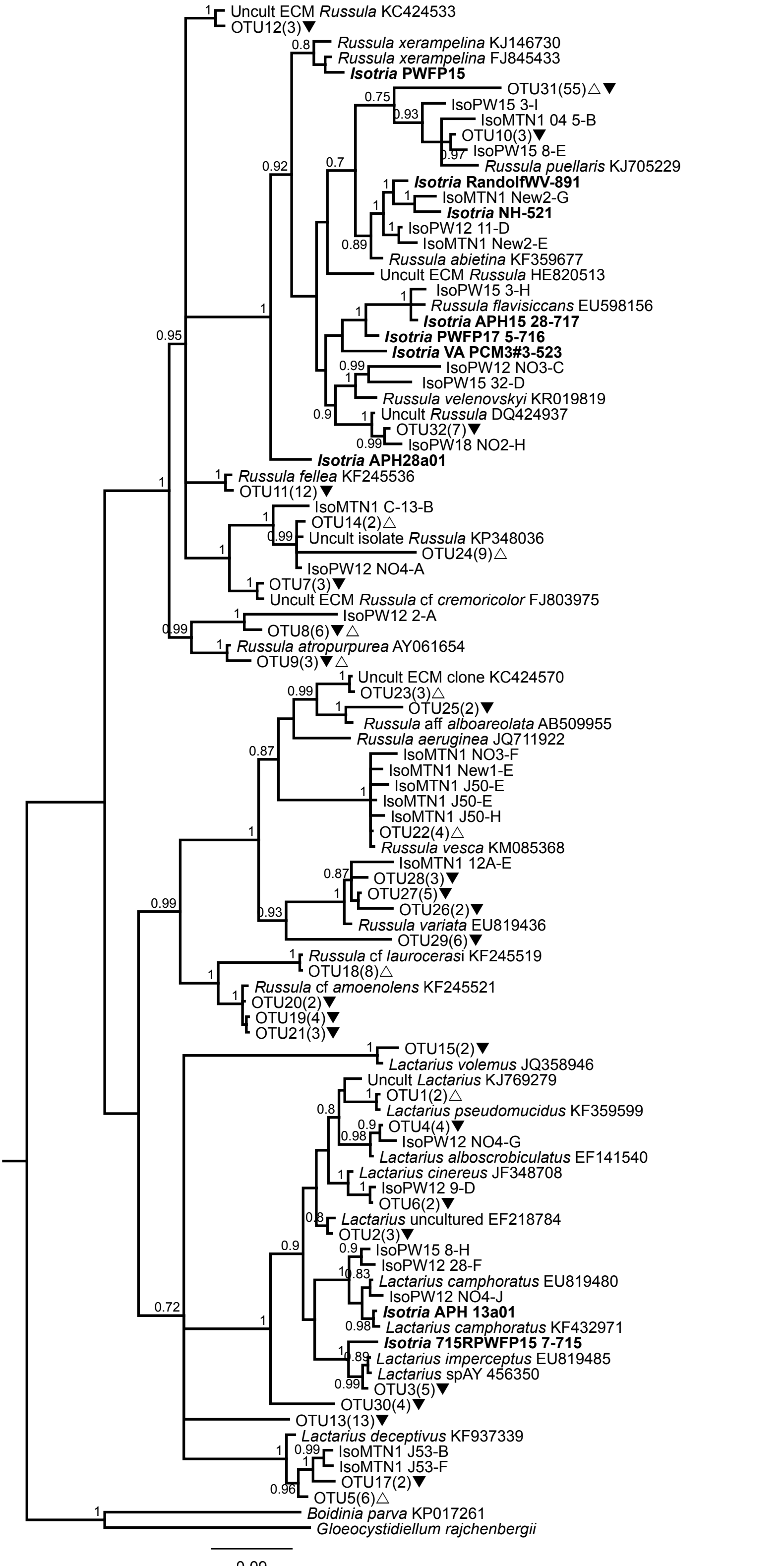

Figure3

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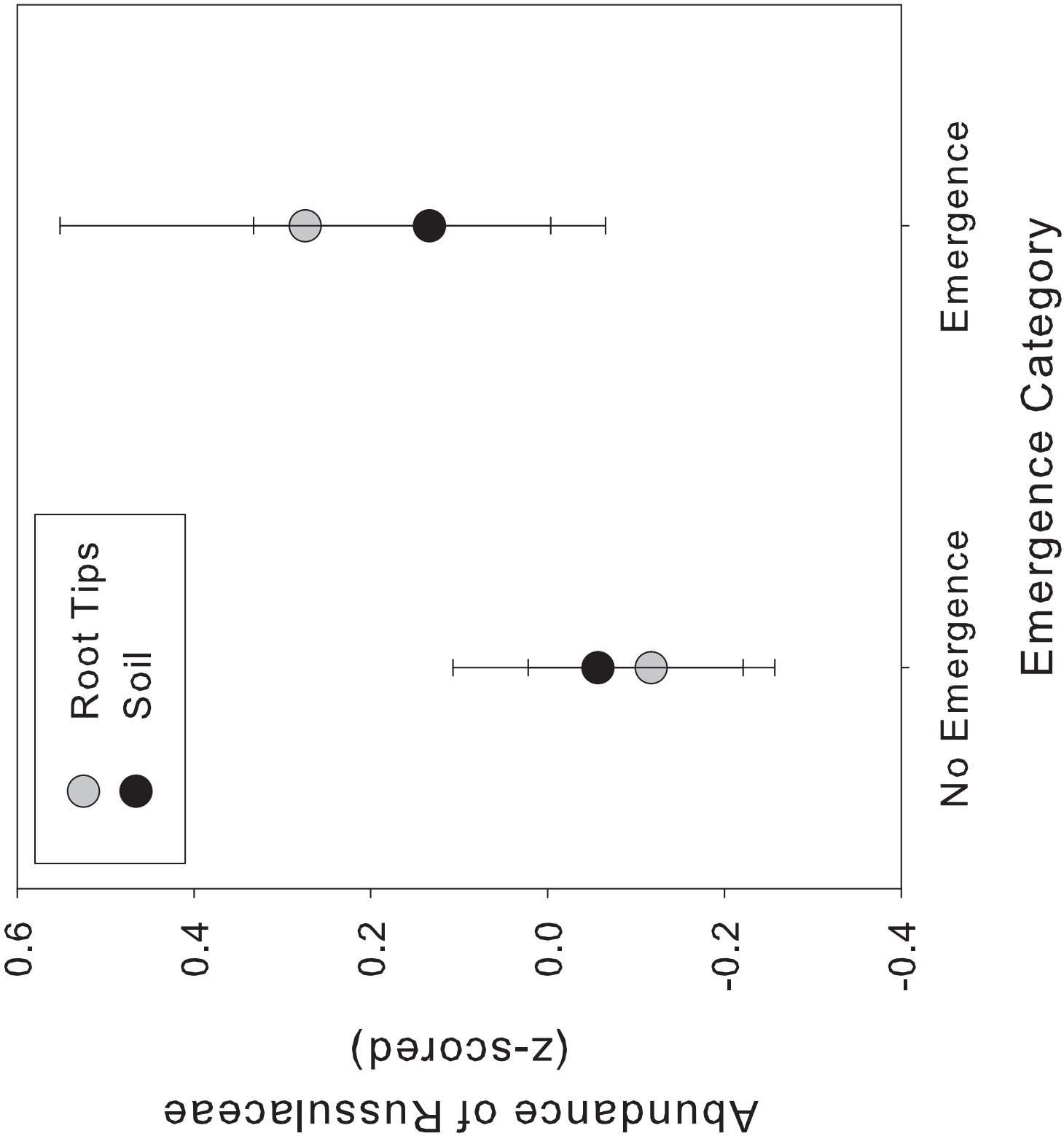

Figure4

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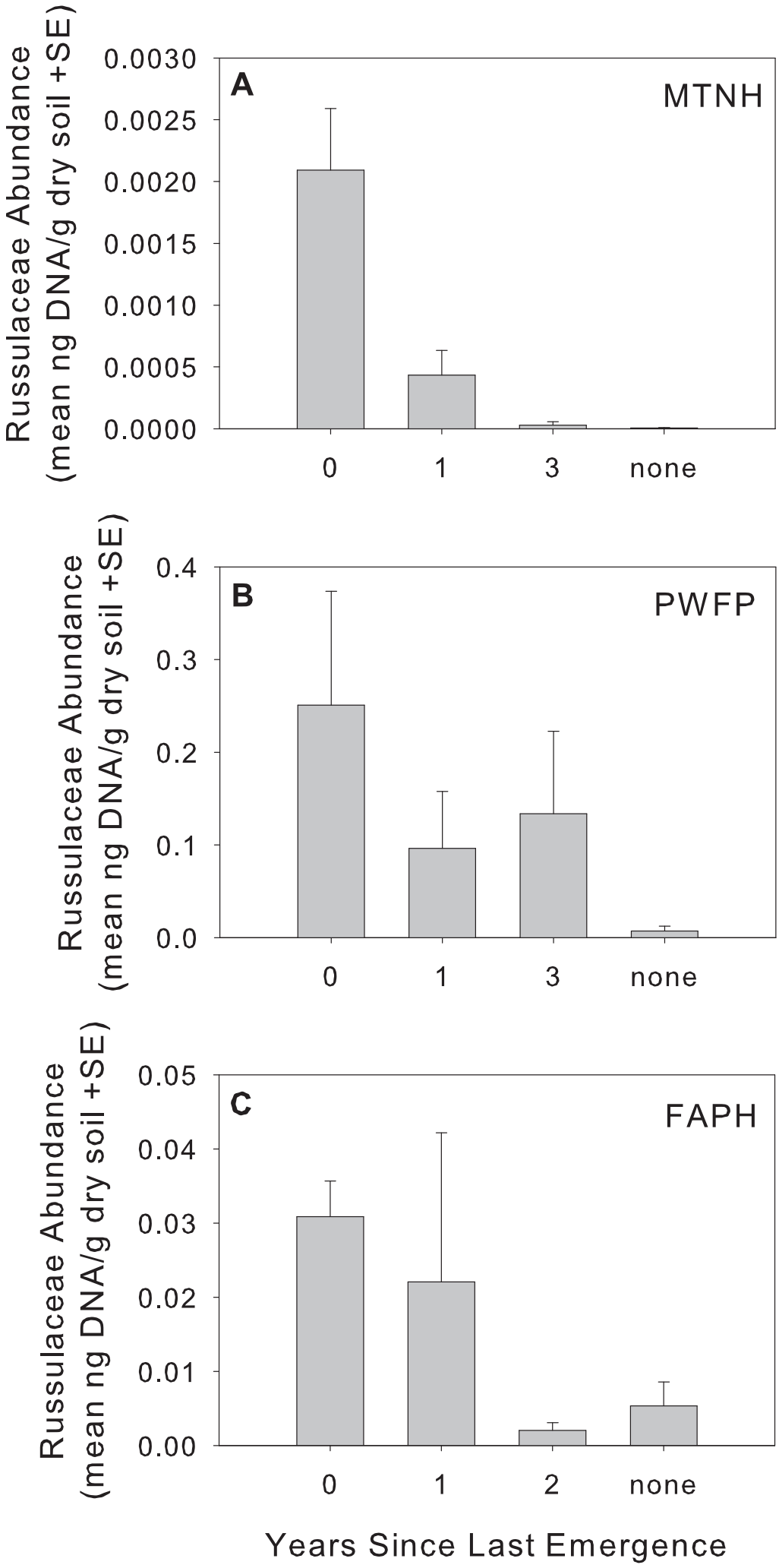

Figure5

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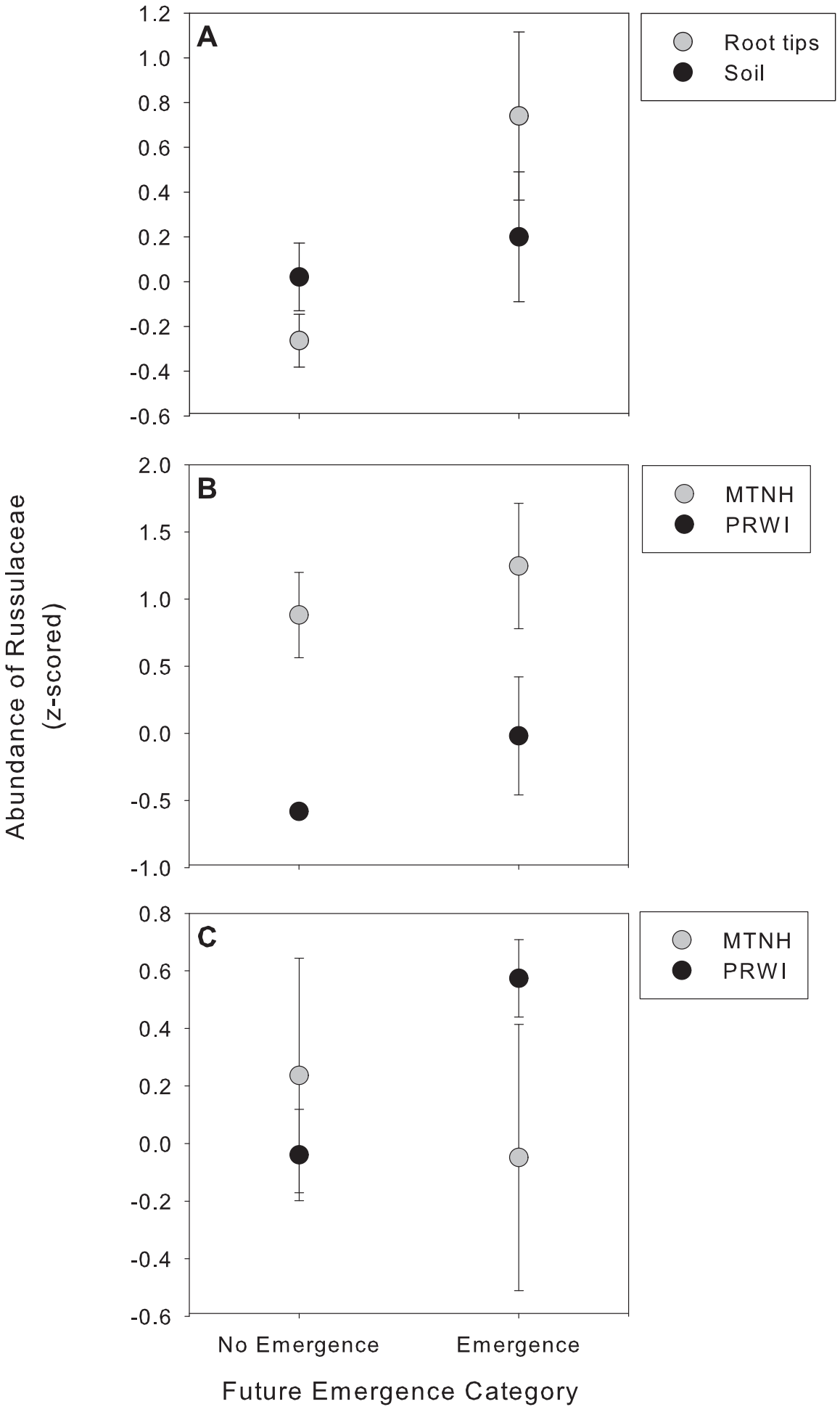

Figure6

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