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PHYLOGENETICS OF CRANICHIDEAE WITH EMPHASIS ON SPIRANTHINAE (ORCHIDACEAE, ORCHIDOIDEAE): EVIDENCE FROM PLASTID AND NUCLEAR DNA SEQUENCES[1]

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DNA sequences from plastid *rbcL* and *matK* genes and the *trnL-F* region, as well as the nuclear ribosomal ITS region, were used to evaluate monophyly and subtribal delimitation of Cranichideae and generic relationships in Spiranthinae. Cranichideae are moderately supported as monophyletic, with Chloraeinae and *Pterostylis-Megastylis* indicated as their collective sisters. Within Cranichideae, Pachyplectroninae and Goodyerinae form a well-supported monophyletic group sister to a ''core spiranthid'' clade that includes, according to their branching order, Galeottiellinae, Manniellinae, and a Prescottiinae-Cranichidinae-Spiranthinae subclade. Inclusion of *Galeottiella* in Spiranthinae, as in previous classifications, renders the latter paraphyletic to all other spiranthid subtribes. Cranichidinae and Spiranthinae (minus *Galeottiella*) are monophyletic and strongly supported, but Prescottiinae form a grade that includes a strongly supported prescottioid Andean clade and a weakly supported *Prescottia*-Cranichidinae clade sister to Spiranthinae. Well-supported major clades in Spiranthinae identified in this study do not correspond to previous alliances or the narrowly defined subtribes in which they have been divided recently. Morphological characters, especially those that have been used for taxonomic delimitation in Cranichideae, are discussed against the framework of the molecular trees, emphasizing putative synapomorphies and problems derived from lack of information or inadequate interpretation of the characters.

Key words: Cranichideae; ITS; *matK*; molecular phylogeny; Orchidaceae; *rbcL*; Spiranthinae; *trnL-F*.

As delimited by Dressler (1993), the tribe Cranichideae Endl. encompasses about 95 genera and 1140 species of predominantly terrestrial orchids distributed in all continents (except in Antarctica), but is especially diverse in the tropical and subtropical regions of America and Asia. Historically, Cranichideae have been placed under various names in the inconsistently delimited subfamilies Neottioideae Lindl. (Lindley, 1840; Bentham, 1881; Schlechter, 1911, 1926; Garay, 1960; Dressler, 1974; Brieger, 1974–1975; Lavarack, 1976; Rasmussen, 1985) or Spiranthoideae Dressler (Dressler, 1979, 1981, 1990, 1993; Burns-Balogh and Funk, 1984, 1986a, b; Szlachetko, 1995). However, recent morphological and molecular phylogenetic studies have provided evidence for their inclusion in an expanded concept of Orchidoideae (Dressler, 1986; Clements, 1995; Dressler and Chase, 1995; Kores et al., 1997, 2000, 2001; Cameron et al., 1999; Freudenstein and Rasmussen, 1999; Freudenstein et al., 2000; cf. Chase et al., 2001).

Cranichideae consist of small plants with fleshy roots, which are fasciculate or sometimes spaced apart along a creeping rhizome, and usually contain spiranthosomes, i.e., a specialized kind of amyloplast that has been found elsewhere in the family only in the mycoheterotrophic Epidendroideae genera *Uleiorchis* and *Wullschlaegelia* (Stern et al., 1993a). Leaves are nonarticulate, soft, with binary or bilobed tracheary elements in the midrib and usually with distinct subsidiary cells (Stern et al., 1993b). Flowers are tubular or less commonly stellate, with more or less erect anther and rostellum, terminal viscidium, and granulate or sectile pollinia (Dressler, 1993). The anthers have endothecial thickenings of types III or IV (according to Freudenstein, 1991). However, all these features are also found in other orchid groups, and Cranichideae lack obvious ''key'' morphological characters permitting their recognition. Phylogenetic analyses of sequences of the plastid gene *rbcL* (Kores et al., 1997; Cameron et al., 1999; Chase et al., 2001) and an intron of the protein-coding mitochondrial gene *nad1* (Freudenstein et al., 2000) have recovered a paraphyletic Cranichideae. However, monophyly of Cranichideae has been indicated by studies based on morphological characters (Freudenstein and Rasmussen, 1999) and plastid *matK* and *trnL-F* DNA sequences (Kores et al., 2000, 2001). Nevertheless, none of those studies has been focused specifically on Cranichideae, and their sampling of this tribe has been limited. Vargas (1997) carried out a preliminary cladistic analysis of Cranichideae based on morphological characters, but his study was hindered by the small sample of both characters and

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taxa considered, inadequate information on some groups, and lack of suitable outgroups.

Dressler (1993) recognized six subtribes in Cranichideae, which agreed with subtribal limits set previously by Schlechter (1926) except for recognition of a new subtribe, Prescottiinae Dressler (Dressler, 1990), to accommodate several genera formerly included in Cranichidinae. Subtribes Goodyerinae Klotzsch and Spiranthinae Lindl. are both widespread (the latter is absent in sub-Saharan Africa), Cranichidinae Lindl. and Prescottiinae are restricted to the Neotropics, and Manniellinae Schltr. and Pachyplectroninae Schltr. are endemic to tropical Africa and New Caledonia, respectively. A different scheme was proposed by Szlachetko (1995), who treated Cranichideae sensu Dressler (1993) as a distinct, narrowly defined subfamily Spiranthoideae containing three tribes, namely, Cranichideae, Goodyereae King & Pantl., and Spirantheae Endl.; these included one, three, and six subtribes, respectively. Throughout this paper, we use the tribal and subtribal concepts of Dressler (1993) unless otherwise indicated.

Generic delimitation in Spiranthinae has been the subject of controversy since Schlechter revised the group in 1920. Schlechter (1920) based his classification mainly on features of the rostellum and viscidium and recognized 24 genera, grouped into four generic alliances (see also Schlechter, 1926). This grouping represented a considerable increase in the number of genera recognized in the subtribe; for instance, Lindley (1840) recognized only four genera (excluding *Cnemidia* Lindl., a synonym of *Tropidia* Lindl.), whereas Bentham (1881) accepted only two. Schlechter's (1920) classification was acrimoniously criticized by some orchid taxonomists for relying on characters ''too recondite for practical purposes'' (Ames, 1922, 1923), and several orchid inventories in Tropical America, where most of the genera occur, used an all-encompassing concept of *Spiranthes* (e.g., Williams, 1951; Ames and Correll, 1952; Schweinfurth, 1958; McVaugh, 1985). Nevertheless, other workers, such as Hoehne (1945), Correa (1955), Brieger (1974–1975), and Garay (1978), followed Schlechter. The controversy acquired a new impetus with the nearly simultaneous publication of two further generic revisions of Spiranthinae by Garay (1982) and Balogh (1982; also Burns-Balogh, 1986), which differed sharply from one another (and from Schlechter's) in the number and circumscription of the genera they recognized. Both new schemes were also largely based on characters of the rostellum and viscidium. Balogh (1982) recognized 16 genera, 11 of which she assigned to four alliances similar to those of Schlechter (1920), but the remaining five were considered to represent the probable remnants of independent evolutionary lines. On the other hand, Garay (1982) accepted 44 genera without recognizing generic alliances. A considerable number of taxonomic papers devoted to Spiranthinae followed the revisions by Garay (1982) and Balogh (1982), many of them proposing new genera (e.g., Burns-Balogh et al., 1985; Burns-Balogh, 1989; Szlachetko, 1991b, d, 1994; Szlachetko and González Tamayo, 1996; Szlachetko et al., 2000). However, those papers, as for most previous taxonomic work on Spiranthinae, relied completely on intuitive weighting of a few floral morphological characters and lacked explicit phylogenetic hypotheses. Only a few studies have explored phylogenetic relationships of individual genera or groups of genera in Spiranthinae (Burns-Balogh and Robinson, 1983; Burns-Balogh, 1988).

In this study, we assess phylogenetic relationships in Cranichideae with nucleotide sequences of plastid and nuclear DNA. The plastid DNA regions analyzed here include the protein-encoding genes *rbcL* and *matK* and part of the *trnK* intron flanking the latter, the *trnL* intron, and the *trnL-trnF* intergenic spacer. The nuclear region studied includes the two internal transcribed spacers (ITS1 and ITS2) and the intervening gene 5.8S of the nuclear ribosomal multigene family. All these regions have been used previously in phylogeny inference at various taxonomic levels in other groups of Orchidaceae (Bateman et al., 1997; Kores et al., 1997, 2000, 2001; Pridgeon et al., 1997, 2001; Aceto et al., 1999; Cameron et al., 1999; Douzery et al., 1999; Ryan et al., 2000; van den Berg et al., 2000; Whitten et al., 2000; Bellstedt et al., 2001; Goldman et al., 2001; Gravendeel et al., 2001; Sosa et al., 2001; Williams et al., 2001a, b), as well as many other angiosperms (reviewed in Soltis and Soltis, 1998). Our aim is to provide a phylogenetic framework to evaluate current classifications of Cranichideae, with a special focus on delimitation of subtribe Spiranthinae. We also carry out the first general evaluation of generic relationships in Spiranthinae, but this aspect will be explored in more detail elsewhere (G. A. Salazar and M. W. Chase, unpublished manuscript).

MATERIALS AND METHODS

Taxon sampling—Fifty species, representing all tribes of subfamily Orchidoideae sensu Chase et al. (2001) were included in this study (listed in Appendix at http://ajbsupp.botany.org/v90/). Forty-two species in 35 genera represent all the subtribes recognized by Dressler (1993) in tribe Cranichideae. Representatives of Chloraeinae, Megastylidinae, Pterostylidinae, Diurideae, Codonorchideae, and Orchideae/Diseae were used as outgroups to evaluate monophyly of Cranichideae. In two instances (*Goodyera* and *Satyrium*), sequences of all the DNA regions analyzed were not available from the same species. Thus, sequences of *rbcL*, *matK-trnK*, and the ITS region of *Goodyera* are of *G. pubescens*, whereas that of *trnL-F* is of *G. viridiflora*. The *matK-trnK* sequence of *Satyrium* is of *S. nepalense* but all the others of *S. rhynchanthum*. About 10% of the sequences were obtained from Genbank, and the remaining 90% were originally produced for this study (see Appendix, http://ajbsupp.botany.org/v90/).

DNA extraction, amplification, and sequencing—Total DNA was extracted from fresh or silica gel-dried plant tissue with a modification of the 2× cetyltrimethylammonium bromide (CTAB) procedure of Doyle and Doyle (1987). DNA was cleaned directly with QIAquick silica columns (Qiagen, Crawley, West Sussex, UK) or precipitated with 100% ethanol at −20°C and purified on a cesium chloride/ethidium bromide density gradient (1.55 g/mL) with subsequent dialysis and removal of ethidium bromide with butanol.

Amplification of all DNA regions was carried out in 100-μL polymerase chain reactions (PCR) including 0.5 μL 5 units/μL of *Taq* DNA-polymerase (Promega, Madison, Wisconsin, USA), 10 μL 10× Mg-free DNA polymerase buffer (Promega), 12 μL 25 mmol/L $MgCl_2$, 2 μL 10 mmol/L each dNTP, 1 μL 0.4% bovine serum albumin (BSA), 1 μL each primer (100 ng/μL), 72.5 μL ddH_2O, and template DNA. Alternatively, 50 μL reactions were carried out with 45 μL 1.1× PCR Master Mix (Advanced Biotechnologies, Epsom, Surrey, UK), including 1.25 units *Taq* DNA polymerase, 75 mmol Tris-HCl (pH 8.8 at 25C), 20 mmol ammonium sulfate, 1.5 (for ITS) or 2.5 mmol (for plastid DNA) $MgCl_2$, 0.01% Tween 20, and 0.2 mmol each dNTP, to which were added 0.5 μL each primer (100 ng/μL), 0.5 μL 0.4% BSA, 2 μL ddH_2O, and template DNA. The PCR mix for amplifying the ITS region included 2% dimethyl sulfoxide (DMSO) to reduce problems related to secondary structure and efficiency of PCR primer binding.

Using primers 1F and 1360R (Kores et al., 1997), the *rbcL* gene was usually amplified as a single piece ca. 1350 base pairs (bp) long. However, for degraded DNA, the same region was amplified in two overlapping fragments with these primers and the internal primers 636F and 724R (Muasya et al., 1998). The PCR profile consisted of an initial 2-min premelt at 94°C and 28–

30 cycles of 1-min denaturation at 94°C, 30-s annealing at 48°C, and 1-min extension at 72°C, followed by a final extension of 7 min at 72°C.

The *matK-trnK* region, including *matK* and the whole 3′ portion of the *trnK* intron (downstream *matK*), was usually amplified as a single fragment ca. 1850 bp long with primers −19F (Molvray et al., 2000) and 2R (Steele and Vilgalys, 1994). Degraded DNA was amplified in smaller fragments with combinations of −19F, 2R, and internal primers 556R, 731F (Molvray et al., 2000), 390F, and 1326R (Cuénoud et al., 2002). The PCR profile consisted of a 2-min 30-s initial premelt at 94°C, 28–30 cycles with 1-min denaturation at 94°C, 1-min annealing at 52°C, a first 2-min 30-s extension at 72°C, increased by 8 s on each consecutive cycle, and final extension of 7 min at 72°C. For amplifying degraded DNA, the annealing temperature was lowered to 48°C and the extension time was not increased on each cycle.

The *trnL-trnF* region, including the intron of *trnL* and the *trnL-trnF* intergenic spacer, was amplified either as a single piece with primers c and f or as two nonoverlapping fragments with primers c–d and e–f (all these from Taberlet et al., 1991). The PCR profile was as for *rbcL*.

The entire ITS region was amplified with primers ITS4 and ITS5 (White et al., 1990) and in some cases with primers 17SE and 26SE (Sun et al., 1994). The PCR profile for ITS5-ITS4 included an initial 2-min premelt at 94°C, 28–30 cycles of 1-min denaturation at 94°C, 1-min annealing at 52°C, and 2-min extension at 72°C, with final extension of 7 min at 72°C. The PCR profile for 17SE–26SE differed only in a lower annealing temperature, 50°C.

The PCR products were cleaned with QIAquick or CONCERT (Life Technologies, Paisley, UK) silica columns according to the manufacturer's protocols and used in cycle-sequencing reactions with the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq DNA polymerase (Applied Biosystems, Warrington, Cheshire, UK). The 10-μL cycle sequencing reactions included 1 μL terminator mix, 3 μL 2.5× cycle sequencing buffer (200 mmol/L trizma base, 5 mmol/L magnesium chloride, pH 9.0), 1 μL primer (5 ng/μL), and 3–5 μL PCR product, topping with ddH_2O as required.

Cycle-sequencing products were cleaned by precipitation in 25 μL 100% ethanol with 1 μL 3 mol/L sodium acetate (pH 4.6) on ice for 30 min, after which they were centrifuged at 13 000 rpm for 25 min. The alcohol/salt mix was discarded, and the pellet subjected to two washes with 300 μL 70% ethanol, each followed by centrifugation at 13 000 rpm for 15 min. Cleaned cycle-sequencing products were allowed to dry overnight at room temperature or dried in the oven at 65°–70°C for 15 min, and then protected from light until analyzed. Both forward and reverse sequences were analyzed on a PE 377 automated sequencer (Applied Biosystems Inc.), and the resulting electropherograms were edited and assembled with Sequencher versions 3.1 or 4.1 (Gene Codes Corp., Ann Arbor, Michigan, USA).

Sequence alignment and indel coding—Sequences of *rbcL* and *matK* were unambiguously aligned by visual inspection. No indels were present in *rbcL*, and only a few were found in *matK*. The 3′ portion of the *trnK* intron and the *trnL-F* and ITS regions were aligned with Clustal W (Thompson et al., 1994) and visually adjusted as necessary, following the guidelines of Kelchner (2000). A total of 344 positions of ambiguous alignment in the *trnK* intron, *trnL* intron, and *trnL-F* intergenic spacer were excluded from the analyses; these represented 6.2% of the positions in the aligned sequences. All nonautapomorphic indels were coded as binary (presence/absence) characters with the simple indel coding method of Simmons and Ochoterena (2000) and appended to the sequence matrices. In total, we coded 15 indels in the *matK-trnK* region, 47 in the *trnL-trnF* region, and 52 in the ITS region. The aligned matrix is available on request from G.A.S. (gasc@servidor.unam.mx) and M.W.C. (m.chase@rbgkew.org.uk).

Phylogenetic analyses—Maximum parsimony analyses were conducted in PAUP* version 4.0b10 (Swofford, 1998) for the 12 data sets indicated in Table 1. Except for the indels-only data set, all analyses consisted of 1000 replicates of random sequence addition with tree bisection-reconnection (TBR) branch swapping and the MULTREES option on, saving all most-parsimonious trees. Analysis of the indels-only data set differed only in that up to 20 trees were saved per replicate (because of the exceedingly large number of trees generated). All characters were unordered and equally weighted (Fitch parsimony; Fitch, 1971). Individual gap positions were treated as missing data, the indels being separately coded and treated as additional characters (see earlier). Internal support of clades was evaluated by the bootstrap (Felsenstein, 1985), with 500 bootstrap replicates with tree bisection-reconnection (TBR) branch swapping, saving up to 10 trees per replicate to reduce time spent swapping on large islands. For the combined analyses of all plastid and nuclear sequences and indels, clade support was also evaluated with the Bremer support (Bremer, 1988). Constraint trees were created with AutoDecay version 4.0.2′ (Eriksson, 1999) and run in PAUP*, with 100 random sequence addition replicates and TBR branch swapping, saving up to 10 trees per replicate. To compare performance of transitions (ts) vs. transversions (tv), the number of tv and their CI and RI were calculated on one of the most parsimonious trees (MPTs) found in the combined analysis by using a step matrix giving zero weight to ts and the TREE SCORES command in PAUP*. The number of ts and their CI and RI were calculated from these data. Number of steps, CI, and RI were calculated for each of the regions sequenced, each of the separate data sets analyzed and, in the case of *rbcL* and *matK*, for first, second, and third codon positions on one of the MPTs of the combined analysis.

An independent, model-based estimate from combined sequence data was generated using Bayesian inference (Larget and Simon, 1999; Lewis, 2001) with the method implemented in MrBayes 2.01 (Huelsenbeck and Ronquist, 2001). The model of sequence evolution chosen was the general time-reversible model (Rodríguez et al., 1990) with a proportion of invariant characters and gamma distribution. This model best fit our combined data set according to a hierarchical likelihood ratio test conducted in Modeltest version 3.06 (Posada and Crandall, 1998). The values of the rate matrix estimated by Modeltest were input in MrBayes, and the frequency of each type of nucleotide was empirically assessed from the data set as part of the analysis. Four Markov chains starting with a random tree were run simultaneously for 200 000 generations, sampling from the trees every 10th generation. Stationarity was reached at around generation 68 000; thus, the first 70 000 generations (7000 trees) were discarded as the ''burn-in,'' and inference about relationships was based only on the remaining 130 000 generations (= 13 000 trees).

RESULTS

Results from the rbcL analysis—The *rbcL* matrix comprised 1236 characters (corresponding to positions 79–1314 of the *rbcL* sequence of *Nicotiana*), of which 181 (14.6%) were variable and 102 (8.2%) were potentially parsimony-informative. The analysis found 2304 MPTs with a length of 329 steps, a CI of 0.61, and an RI of 0.75. Figure 1 shows the strict consensus of the 2304 trees, indicating the bootstrap percentages of the clades. Although several groups are resolved, few of them are supported by high bootstrap percentages (BP). The *rbcL* analysis does not provide evidence for monophyly of Cranichideae because clades Goodyerinae-Pachyplectroninae (A; BP 54) and ''core spiranthids'' (B–J; BP 71) are not resolved from *Diuris*, Chloraeinae, *Megastylis*, and *Pterostylis*. Goodyerinae (*Goodyera* through *Ludisia* in clade A) are strongly supported (BP 92), and so are *Ponthieva* (BP 100) and *Spiranthes* sensu stricto (BP 94). Cranichidinae (E) are monophyletic but weakly supported (BP 65) and Prescottiinae (C–D) are unresolved, except for a weakly supported clade including *Aa* and *Porphyrostachys*. Spiranthinae sensu Dressler (1993) are paraphyletic to Manniellinae, Cranichidinae, and Prescottiinae because of the position of *Galeottiella*, which is sister to all other core spiranthids; the latter (B–J in Fig. 6) are monophyletic and moderately supported by *rbcL* alone (BP 79). However, *Manniella* is paraphyletic, although the association of *M. gustavi* with the Prescottiinae-Cranichidinae-Spiranthinae clade (C–J) received only a low BP (56). Spiranthinae are monophyletic (BP 84) if *Galeottiella* is excluded, and all major clades identified within Spiranthinae minus *Galeot-*

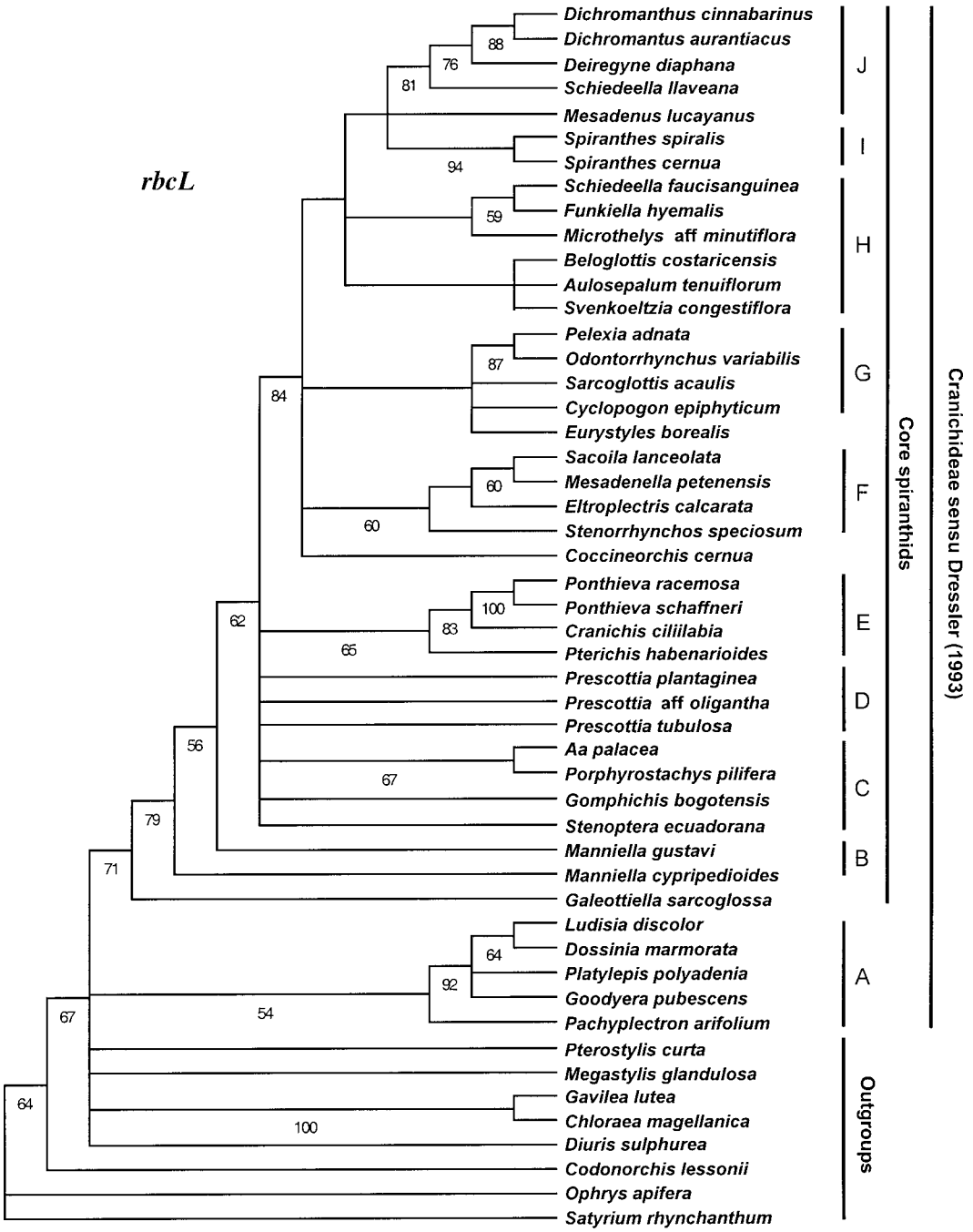

Fig. 1. Strict consensus of 2304 trees from the parsimony analysis of *rbcL* (length = 329 steps, CI = 0.61, RI = 0.75). Bootstrap percentages >50 are indicated below the branches.

tiella (F–J, *Coccineorchis*, and *Eurystyles*) by *rbcL* were also recovered, with minor variations, by all the other data sets.

Results from the matK-trnK analysis—The aligned matrix of the *matK-trnK* region comprised 1900 aligned positions, 1632 of which corresponded to *matK* and 268 to the 3′ portion of the *trnK* intron. Of the 1900 sites, 692 (36%) were variable, and 423 (22%) were potentially parsimony-informative. Analysis found 18 MPTs with a length of 1617 steps, CI of 0.57, and RI of 0.68. The addition of the 15 indels resulted in 18 MPTs with a length of 1646 steps and the same CI and RI. The strict consensus of the latter trees is depicted in Fig. 2. The topology of the strict consensus obtained without the indels is identical, but the BPs of some clades are slightly lower.

This region produced considerably more resolution and more well-supported groups (i.e., BP $\geq$ 90) than *rbcL*, although there are many clades in common. Cranichideae are not resolved as monophyletic in the *matK-trnK* analysis because strongly supported clades Pachyplectroninae-Goodyerinae (A; BP 100) and core spiranthids (*Galeottiella* + B–J; BP

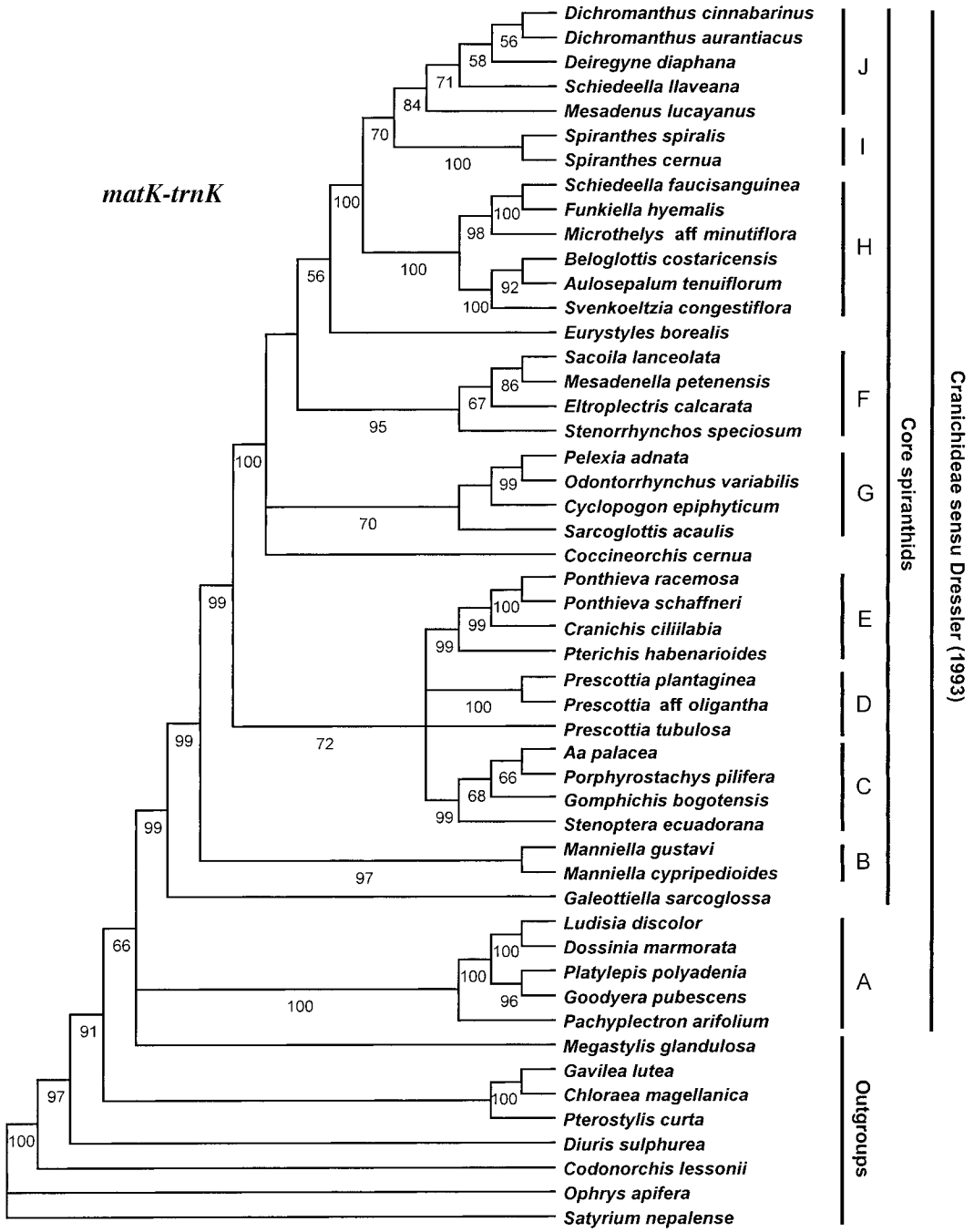

Fig. 2. Strict consensus of six trees from the parsimony analysis of the *matK-trnK* region, including indels (length = 1646 steps, CI = 0.57, RI = 0.68). Bootstrap percentages >50 are indicated below the branches.

99) form a weakly supported trichotomy with *Megastylis*. Within core spiranthids, *Galeottiella* is sister to the rest and a monophyletic Manniellinae (B; BP 97) diverge next. The members of Prescottiinae and Cranichidinae form a weakly supported polytomy (C–E; BP 72), which includes a clade with predominantly Andean Prescottiinae genera (C; BP 99), another formed by *Prescottia plantaginea* and *P.* affinis *oligantha* (BP 100), monophyletic Cranichidinae (E; BP 99), and *P. tubulosa*. Thus, neither *Prescottia* nor Prescottiinae are supported as monophyletic. Spiranthinae minus *Galeottiella* are monophyletic and well supported (*Coccineorchis* + G–J; BP 100), comprising several clades. These include a weakly supported clade (G) with *Sarcoglottis* through *Pelexia*, and the latter strongly supported as sister to *Odontorrhynchus* (BP 99), a strongly supported clade (F) with *Stenorrhynchos* through *Sacoila* (BP 95), and another strongly supported clade (H–J; BP 100), which in turn comprises two subclades. The first of these is weakly supported and includes *Spiranthes* sister to *Mesadenus* through *Dichromanthus* (I–J; BP 70). The second (H) is strongly supported (BP 100) and includes *Svenkoeltzia* sister

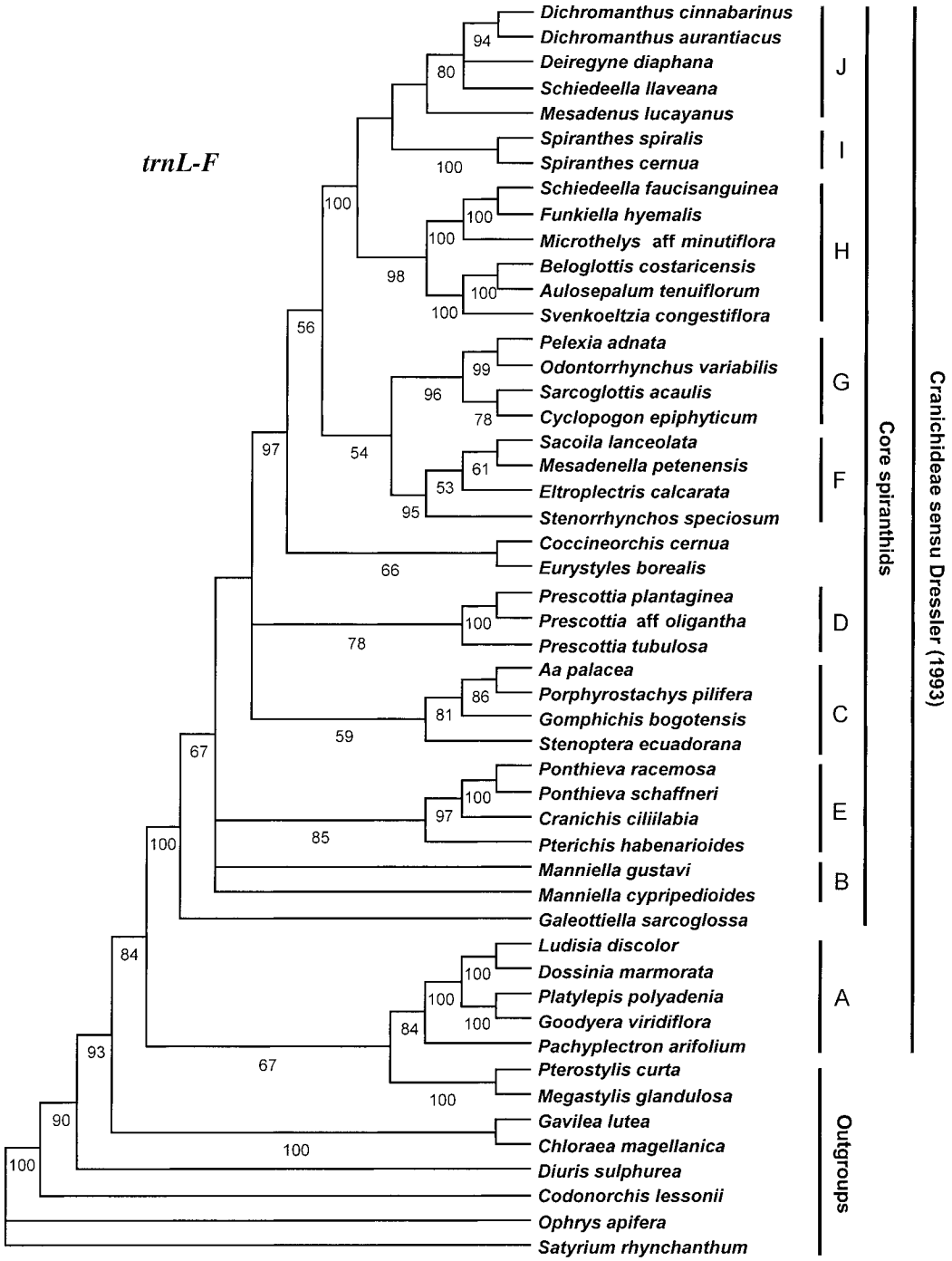

Fig. 3. Strict consensus of 12 trees from the parsimony analysis of the *trnL-trnF* region, including indels (length = 1336 steps, CI = 0.58, RI = 0.69). Bootstrap percentages >50 are indicated below the branches.

to *Aulosepalum* and *Beloglottis*, and *Microthelys* sister to *Funkiella* and *Schiedeella faucisanguinea*; all these relationships are supported by high BP.

Results from the trnL-trnF region analysis—The *trnL* intron and *trnL-trnF* intergenic spacer sequences were analyzed together and comprised 1349 aligned positions (820 and 529, respectively) and 47 indels. Of the 1349 characters, 534 (40%) were variable, and 309 (22%) were potentially parsimony-informative. Analysis without the indels produced six most-parsimonious trees 1237 steps in length, CI of 0.59, and RI of 0.68. The addition of the indels increased the number of shortest trees found to 12, with a length of 1336 steps, CI of 0.58, and RI of 0.69. Both analyses resulted in similar topologies, but the inclusion of the indels increased slightly the BP of some clades. The strict consensus of the four trees found by the analysis that included the indels is depicted in Fig. 3; it shows a paraphyletic Cranichideae in which the strongly sup-

ported *Megastylis-Pterostylis* clade (BP 100) is weakly supported (BP 67) as sister to the *Pachyplectron*-Goodyerinae clade (A). These two clades are collective sisters to core spiranthids with moderately high support (BP 84). In other respects, the patterns found by *trnL-trnF* are similar to those recovered by *matK-trnK* except for lack of support for monophyly of Manniellinae and the absence of a clade that includes all the representatives of Cranichidinae and Prescottiinae (compare C–E in Figs. 2 and 3). *Coccineorchis* and *Eurystyles* form a weakly supported clade (BP 66) that was otherwise recovered solely by the indels-only data set (see later).

Results from the analysis of all plastid regions combined—The combined plastid matrix consisted of 4485 aligned nucleotide positions and 52 indels, and analysis resulted in two MPTs with a length of 3334 steps, a CI of 0.57, and a RI of 0.69 (not shown). Cranichideae are monophyletic but weakly supported (BP 69). Other relationships are most similar to those recovered by the *matK-trnK* region, and in general the support of the clades increased, except for the clade containing all representatives of Prescottiinae and Cranichidinae, which obtained a BP < 50.

Results from the ITS region analysis—The ITS region included a total of 731 aligned positions, of which 479 (65%) were variable and 376 (51%) were potentially parsimony-informative. Internal transcribed spacer 1 and ITS2 consisted of 287 and 279 aligned positions, respectively. The 5.8S gene consisted of 164 bp in most taxa, but in *Pachyplectron* it had an additional base at the 3′ end. Analysis without indels found four MPTs with a length of 1993 steps, CI of 0.44, and RI of 0.62. The addition of the 52 indels increased the number of MPTs to six of 2084 steps long, CI of 0.44, and RI of 0.63. The strict consensus tree in both cases was identical but, as in the analyses of the plastid data sets, BP generally increased slightly with the inclusion of the indels (Fig. 4). Only in the case of the clade including *Cyclopogon* through *Pelexia* (G) was the increase substantial (BP 79 to 91), whereas support for the Pachyplectroninae-Goodyerinae clade (A) decreased from BP 74 to 64.

In the ITS trees, the positions of *Codonorchis* and *Diuris* are inverted with respect to those they occupy in the plastid trees. Thus, *Codonorchis* is sister, with moderate support (BP 82), to all the other taxa except *Diuris*, *Satyrium*, and *Ophrys*. *Pterostylis* is sister to a well-supported clade (BP 97) with Chloraeinae sister to a moderately supported *Megastylis*-Cranichideae clade (BP 78). Cranichideae are monophyletic but obtained BP < 50. There is also little support (BP 64) for a *Pachyplectron*-Goodyerinae clade (A, Fig. 4). In general, however, groups that received support in the analyses of plastid data sets were also recovered by analysis of the ITS region. These include core spiranthids (B–J), Manniellinae (B), Prescottiinae-Cranichidinae-Spiranthinae (D–J), and the latter plus Manniellinae. Prescottiinae are paraphyletic to monophyletic Cranichidinae (E; BP 78) and Spiranthinae (F–J; BP 100); a moderately supported lineage including *Stenoptera* through *Aa* (C) is sister to a weakly supported clade including *Prescottia*-Cranichidinae and Spiranthinae. The same major clades within Spiranthinae found by the plastid data sets were present in the ITS trees, although with a shift in the position of *Spiranthes* (BP < 50).

Results from the analysis of the indels only—This analysis found 18 842 MPTs with a length of 212 steps, CI of 0.54, and RI of 0.78. The strict consensus includes all major clades found by the other data sets but, not surprisingly, shows less internal resolution in most of them (Fig. 5). However, several clades obtained somewhat higher BP than in the *rbcL* tree (see Table 1).

Results from the combined analyses of plastid and nuclear data sets—In comparing the strict consensus trees and the BP of the clades recovered in the separate analyses (see Figs. 1–5), we did not discover strongly supported but conflicting ingroup clades. Therefore, topological differences between the trees produced by these data sets appear to have resulted from insufficient phylogenetic signal in the data sets to resolve certain portions of the tree, i.e., they are sampling errors and represent ''soft incongruences'' (Seelanan et al., 1997). Strongly supported alternative resolutions between plastid and ITS data sets for the positions of some of the outgroup taxa, including that of *Codonorchis* relative to *Diuris* and that of *Pterostylis* relative to *Megastylis* and Chloraeinae, cannot be addressed from the taxonomic sample used for this analysis (cf. Barriel and Tassy, 1998). Moreover, such differences, localized among the outgroups, are unlikely to affect the generally congruent ingroup relationships when the data sets are analyzed in combination (see Wiens, 1998).

Parsimony analysis of all DNA sequences combined produced two MPTs with a length of 5236 steps, CI of 0.52, and RI of 0.65. The addition of the indels resulted in three MPTs with a length of 5455 steps, a CI of 0.52, and a RI of 0.66. The topology of the strict consensus was the same in both cases. The three trees found by the analysis that included the indels differ from one another only in the relationships between the three major clades of Spiranthinae, but none of the three resolutions obtained a high score for any of the measures of support calculated by us (Fig. 7). One such trees, depicted in Figs. 6–7, is topologically identical with the majority-rule consensus of the 13 000 trees retained from the Bayesian analysis; on that tree, branch length, Bremer support (BS), bootstrap percentage (BP), and posterior probability (PP) are indicated for each clade.

It is worth noting that the Bayesian analysis estimated a high posterior probability (PP 0.98–1) for most clades (Figs. 6–7), except for Cranichidinae sister to *Prescottia* (D–E, Fig. 6; PP 0.53), Cranichidinae-*Prescottia* sister to Spiranthinae (D–J, Fig. 6; PP 0.94), *Coccineorchis* sister to the *Pelexia* clade (G, Fig. 7; PP 0.79), and *Coccineorchis* through *Pelexia* collectively sister to the *Stenorrhynchos* clade (F–G, Fig. 7; PP $= 0.54$).

In the combined analysis (Fig. 6), *Codonorchis* is sister to a weakly supported clade (BS 6, BP 76) with *Diuris* and a strongly supported monophyletic group (BS 52, BP $= 100$) including Chloraeinae (BS 51, BP $= 100$), *Pterostylis-Megastylis* (BS 6, BP $= 76$), and a weakly supported, monophyletic Cranichideae (BS 3, BP 71). A sister group relationship between the *Pterostylis-Megastylis* clade and Cranichideae received low support (BS 2, BP 52), but obtained a high posterior probability (PP 1; see Fig. 6). Within Cranichideae, clades *Pachyplectron*-Goodyerinae (BS 19, BP 100), Goodyerinae (BS 52, BP 100), core spiranthids (BS 42, BP 100), Manniellinae (BS 10, BP 98), and a Prescottiinae-Cranichidinae-Spiranthinae clade (BS 18, BP 100) are all strongly supported, as is *Galeottiella* as sister to other core spiranthids

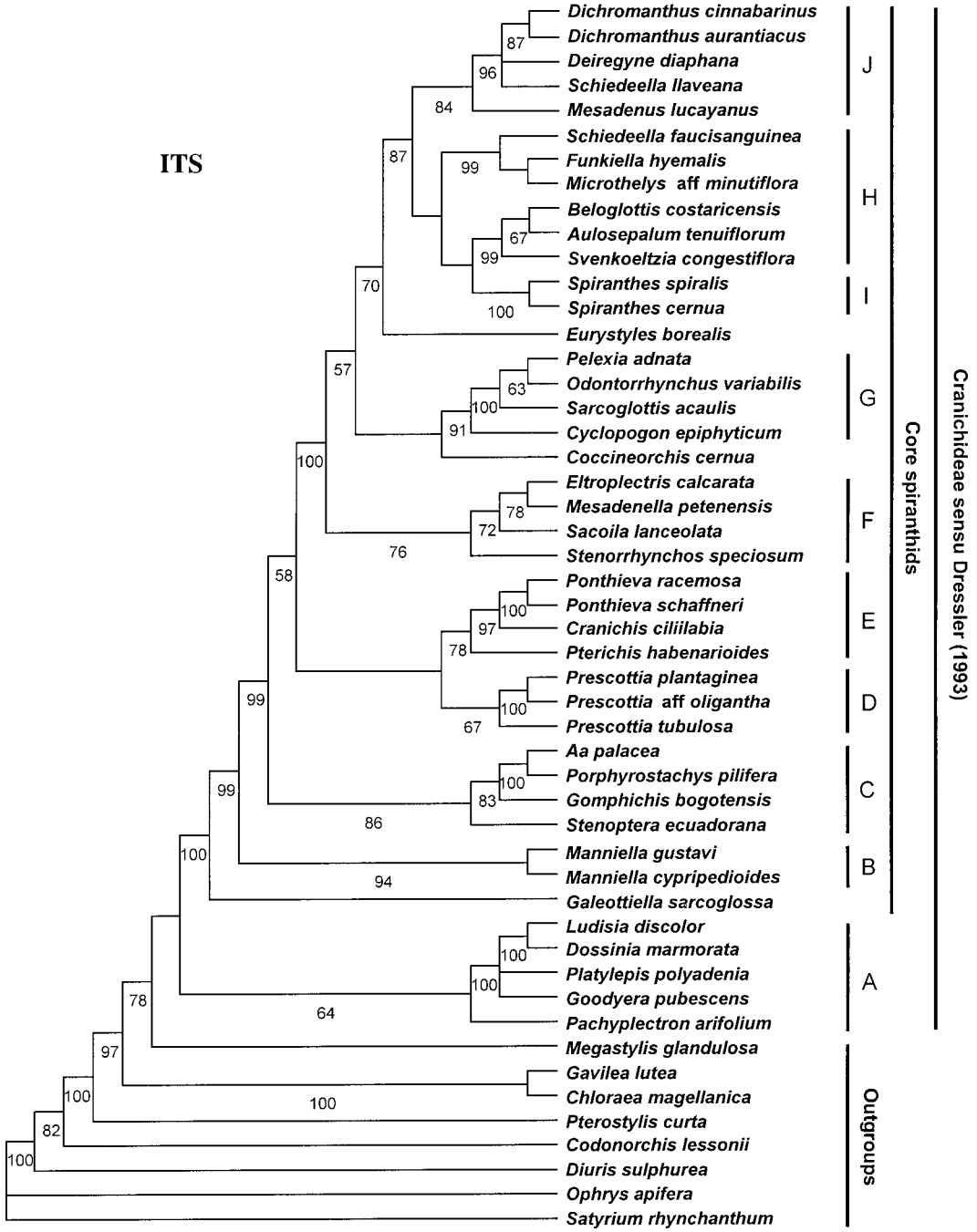

Fig. 4. Strict consensus of six trees from the parsimony analysis of the ITS region, including indels (length = 2084 steps, CI = 0.44, RI = 0.63). Bootstrap percentages >50 are indicated below the branches.

(Fig. 6). Prescottiinae are paraphyletic to Cranichidinae and Spiranthinae, including a well-supported Andean prescottioid clade (C), a moderately supported, monophyletic *Prescottia*, and strongly supported Cranichidinae and Spiranthinae; however, a sister group relationship between Cranichidinae and *Prescottia* has a low posterior probability (PP 0.53), collapsed in trees only two steps longer than the MPTs, and was recovered in less than 50% of the bootstrap replicates. Likewise, Cranichidinae-*Prescottia* sister to Spiranthinae received only low support (BS 2, BP 67). The combined analysis recovered the same three major clades of Spiranthinae found by the separate analyses (F, G, and H–J in Figs. 1–5, 7). *Coccineorchis* was weakly supported to have an association with the *Pelexia* clade (G; BS 1, BP 52), whereas *Eurystyles* obtained low support as sister to the *Spiranthes* clade (I–J; BS 3, BP 63).

DISCUSSION

Molecular evolution—Tables 2–4 show some attributes related to the molecular evolution of the DNA regions analyzed in this study. Previous phylogenetic analyses have indicated an apparent loss of function for *matK* in orchids based on

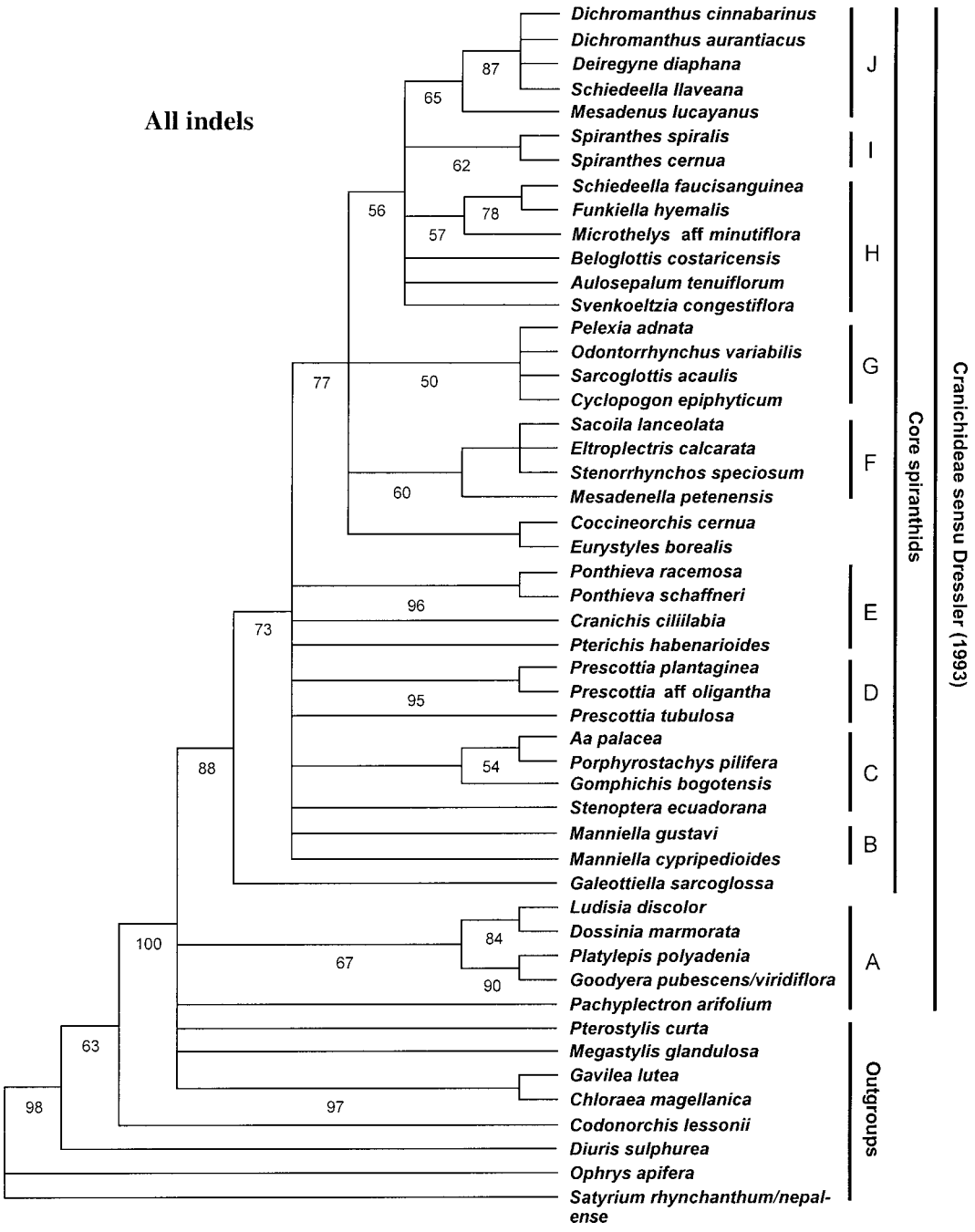

Fig. 5. Strict consensus of 18842 trees from the parsimony analysis of the indels only (length = 212 steps, CI = 0.53, RI = 0.78). Bootstrap percentages >50 are indicated below the branches.

indels that result in loss of a reading frame, a substantially lower proportion of substitutions at third codon positions, an excess of transversions over transitions in comparison with other genes, and internal stop codons (Jarrell and Clegg, 1995; Kores et al., 2000, 2001; Whitten et al., 2000; Goldman et al., 2001; Gravendeel et al., 2001; Pridgeon et al., 2001). Reported ts/tv ratios in orchids vary from 0.66 to 1.02 (Whitten et al., 2000; Goldman et al., 2001; Gravendeel et al., 2001; Pridgeon et al., 2001). The ts/tv ratio of the *matK* sequences analyzed for this study (1.16; Table 4) is the highest reported for orchids, being similar to that reported for some eudicots (e.g., Cornaceae; Xiang et al., 1998). Similarly, the proportion of change at the third codon position (Table 2) is higher than in most other orchids (except Coelogyninae; cf. Gravendeel et al., 2001) but similar to that of some Saxifragaceae, Polemoniaceae, and Poaceae (see Young and dePamphilis, 2000, and references therein). On the other hand, of the 50 *matK* sequences analyzed for this study, only those of *Megastylis glandulosa* and *Dossinia marmorata* included one indel each that resulted in a reading frame shift, but internal stop codons were detected in several others (although BLAST [Altschul et al., 1997] searches in Genbank identified maturase-like conserved

TABLE 1. Characteristics of the data sets analyzed in this study.

	No. characters	No. variable/ informative characters	No. most-parsimonious trees	No. steps (tree length)	CI/RI[a]	Clades with bootstrap percentage ≥90
rbcL sequences	1236	181/102	2304	329	0.61/0.75	4
matK-trnK sequences	1900	691/423	18	1617	0.57/0.68	28
matK-trnK sequences and indels	1915	706/438	18	1646	0.57/0.68	27
trnL-F sequences	1349	534/309	6	1237	0.59/0.68	20
trnL-F sequences and indels	1396	581/356	12	1336	0.58/0.69	24
All plastid sequences	4485	1406/834	2	3207	0.58/0.68	32
All plastid sequences and indels	4547	1468/896	2	3334	0.57/0.69	35
ITS sequences	731	479/376	4	1993	0.44/0.62	20
ITS sequences and indels	783	531/428	6	2084	0.44/0.63	21
All indels	114	114/114	18 842	212	0.54/0.78	6
All sequences combined	5216	1885/1210	2	5236	0.52/0.65	35
All sequences and indels	5330	1999/1324	3	5455	0.52/0.66	35

[a] CI/RI, consistency index/retention index.

domains in the various amino acid sequences of Cranichideae we compared). All the previous information indicates that, even if *matK* is a pseudogene in Cranichideae, it retains much of its structure and evolves in a similar fashion to other angiosperms (e.g., Hilu and Liang, 1997; Young and dePamphilis, 2000).

Limits and relationships of Cranichideae—Recent anatomical (Stern et al., 1993a, b), embryological (Clements, 1995) and molecular studies (Kores et al., 1997, 2000, 2001; Cameron et al., 1999; Freudenstein et al., 2000; Chase et al., 2001) have helped to clarify greatly the relationships of Cranichideae, showing that they are not closely related to tribes Diceratosteleae Dressler and Tropidieae Dressler, with which they have been associated in Spiranthoideae (Dressler, 1979, 1981, 1993; Burns-Balogh and Funk, 1986a, b). Those studies have also shown that Cranichideae occupy a derived position within subfamily Orchidoideae (e.g., Chase et al., 2001; Kores et al., 2001); therefore, raising Cranichideae sensu Dressler (1993) to subfamily status, as proposed by Szlachetko (1995), is taxonomically superfluous and results in paraphyly of Orchidoideae.

In this study, Cranichideae sensu Dressler (1993) were found to be monophyletic with moderately low support (BS 3, BP 71; Fig. 6), and Chloraeinae and a *Pterostylis-Megastylis* clade were strongly supported as collective sisters to Cranichideae, in agreement with previous analysis of plastid DNA sequences (Kores et al., 2000, 2001; Chase et al., 2001) and embryology (Clements, 1995, pro parte). However, there is low support for *Pterostylis-Megastylis* being sister to Cranichideae, and the relationships of these groups to each other and to Chloraeinae should be further investigated by including a broader sample of representatives of *Megastylis*, *Pterostylis*, and Chloraeinae. Chase et al. (2001) suggested that Chloraeinae, Pterostylidinae, and Pachyplectroninae may deserve to be raised to tribal status and, in fact, labeled the corresponding clades in their tree as Chloraeeae, Pterostylideae, and ''Pachyplectroneae,'' although the last name has not yet been validly

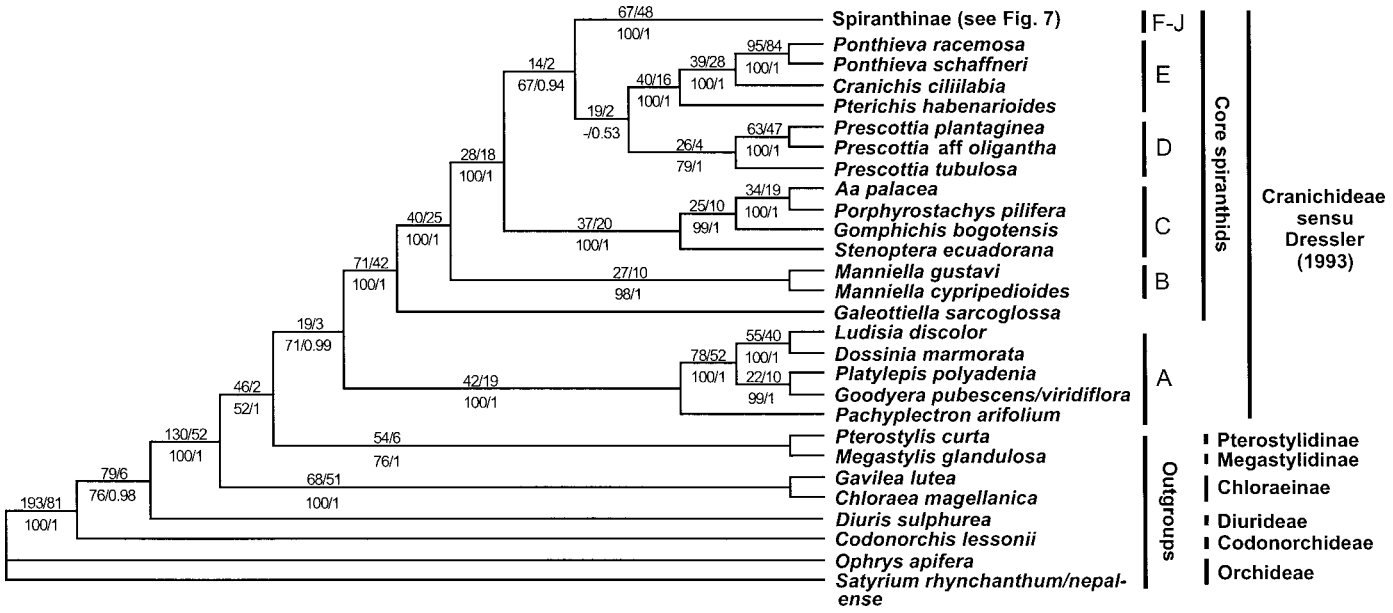

Fig. 6. One of the three most-parsimonious trees of the combined analysis of *rbcL, matK-trnK, trnL-trnF,* and ITS sequences and indels (length = 5455 steps, CI = 0.52, RI = 0.66). Branch length/Bremer support and bootstrap percentage (>50)/posterior probability (from the Bayesian analysis) are indicated above and below the branches, respectively. This tree and the Bayesian majority-rule probability tree have identical topologies.

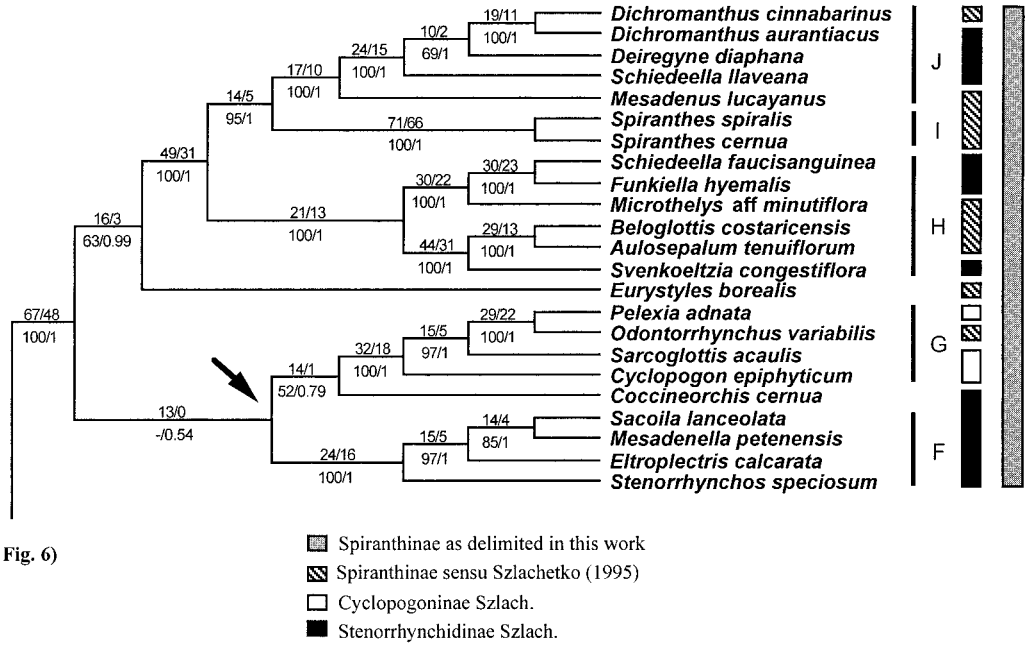

Fig. 7. Continuation of the most-parsimonious tree in Fig. 6. Bars compare the subtribes recognized by Szlachetko (1995) with the relationships found in this study (see text). Arrow indicates groups absent in the strict consensus.

published at tribal rank. Our results indicate that *Pachyplectron* belongs in Cranichideae and is most closely related to Goodyerinae (see later) and are consistent with previous suggestions by Clements (1995) and Kores et al. (2000, 2001) to include Chloraeinae, Megastylidinae, and Pterostylidinae in an expanded concept of Cranichideae.

Cranichideae sensu Dressler (1993) can be diagnosed by the presence of spiranthosomes (Stern et al., 1993a, b; Stern, 1999) and the possession of endothecial thickenings of types III or IV (Freudenstein, 1991). Other anatomical features that have been considered distinctive of Cranichideae are more widespread, although they may provide evidence of a relationship at other taxonomic levels (cf. Fig. 8). For instance, binary or bilobed xylary elements in the leaf midrib distinguish Cranichideae from Diceratosteleae and Tropidieae (see earlier) but have also been found in *Megastylis glandulosa* (Pridgeon, 2001), *Pterostylis curta*, and some Diurideae, such as *Coilochilus neocaledonicus* and *Diuris sulphurea* (G. A. Salazar, personal observation), and could represent a synapomorphy of the Diurideae-Cranichideae (sensu lato) clade. Mesoperigenous stomatal development was formerly thought to be restricted among orchids to Cranichideae, but apparently it is present also in Orchideae (Rasmussen, 1981; Dressler, 1993). Several morphological characters putatively synapomorphic for various clades identified in this study are shown in Fig. 8.

TABLE 2. Number of steps, CI, and RI for each codon position of *rbcL* and *matK* (ACCTRAN optimization on the MPT of the combined analysis, Figs. 6–7).

Gene/codon position	No. steps (rate)[a]	CI	RI
rbcL			
1	62 (1.8)	0.61	0.66
2	33 (1.0)	0.55	0.69
3	240 (7.3)	0.61	0.76
matK			
1	421 (1.2)	0.60	0.68
2	336 (1.0)	0.57	0.67
3	623 (1.8)	0.53	0.67

[a] Standardized to second position.

Subtribal relationships—Our study found support for monophyly of some, but not all, of the subtribes recognized in Cranichideae by Dressler (1993); subtribal limits and relationships within core Cranichideae are discussed in the following sections, according to their branching order in the tree depicted in Fig. 6.

Pachyplectroninae and Goodyerinae—Schlechter (1911) included *Pachyplectron* in Cryptostylidinae but subsequently established for it the monotypic subtribe Pachyplectroninae (Schlechter, 1926). Brieger (1974–1975) placed *Pachyplectron* in his alliance ''Viscidifera'' in subtribe Chloraeinae, included in Spirantheae (= Cranichideae). Dressler and Dodson (1960) considered the affinities of *Pachyplectron* as uncertain, although they mentioned a possible relation to Diuridinae and Cryptostylidinae, but later Dressler (1974) included it in Cranichideae as a member of Goodyerinae. However, subsequent classifications (Dressler, 1981, 1990, 1993; Burns-Balogh and Funk, 1986a, b; Szlachetko, 1995) have maintained Pachyplectroninae.

Our analyses strongly support *Pachyplectron* as sister to Goodyerinae (Fig. 6), in agreement with previous molecular studies (Kores et al., 2000, 2001). Szlachetko (1995) noticed the presence in *Pachyplectron* of a spur ''typical for Goodyerinae,'' but nevertheless considered the former to stand in an isolated position. In light of the relationships found in this study, the shared possession of a labellar spur, otherwise unknown in Cranichideae, should be considered a synapomorphy for the Pachyplectroninae-Goodyerinae clade. Goodyerinae, in

TABLE 3. Patterns of nucleotide substitution for DNA regions included in this study (ACCTRAN optimization on the MPT of the combined analysis, Figs. 6–7).

	rbcL	*matK*	*trnK* intron	*trnL* intron	*trnL-trnF* spacer	ITS1	5.8S	ITS2
No. sites	1236	1602	298	820	529	286	165	279
No. (percentage) variable sites	181 (14.6)	579 (36.1)	113 (37.9)	295 (36.0)	240 (45.4)	221 (77.3)	40 (24.2)	218 (78.1)
No. (percentage) informative sites	102 (8.2)	362 (22.6)	61 (20.5)	158 (19.3)	151 (28.5)	181 (63.3)	19 (11.5)	176 (63.1)
No. steps (substitutions)	335	1372	257	688	562	1032	76	916
Mean no. steps per site	0.27	0.86	0.86	0.84	1.06	3.61	0.46	3.28
Mean no. steps per variable site	1.85	2.37	2.27	2.33	2.34	4.67	1.9	4.20
Mean no. steps per informative site	3.28	3.79	4.21	4.35	3.72	5.70	4	5.20
CI	0.60	0.56	0.60	0.57	0.61	0.41	0.60	0.44
RI	0.74	0.67	0.70	0.64	0.70	0.61	0.59	0.60

turn strongly supported as monophyletic by our data, can be diagnosed by the possession of a creeping rhizome and sectile pollinia (Fig. 8). Szlachetko (1991c, 1995) divided Goodyerinae into three subtribes, but splitting this natural group only results in loss of phylogenetic information and unnecessary inflation of nomenclature. Moreover, the close relationship between *Pachyplectron* and Goodyerinae would support the former being accommodated in an expanded concept of the latter, as in Dressler (1974).

Galeottiellinae—Galeottiella, previously considered as a member of Spiranthinae (Schlechter, 1920; Balogh, 1982; Garay, 1982; Burns-Balogh, 1986; Szlachetko, 1991a, 1995), was established by Schlechter (1920) to include a species originally described as *Spiranthes sarcoglossa* A. Rich. & Galeotti. *Galeottiella* was distinguished from other Spiranthinae mostly because of its distinctive habit, consisting of an upright stem along which the leaves are spirally arranged, and floral features such as the lateral sepals being connate for about one-third of their length, the fleshy, concave labellum, and the bluntly bilobed rostellum, short anther, and short, thick viscidium (Schlechter, 1920). Balogh (1982; also as Burns-Balogh, 1986) reduced *Galeottiella* to a section of *Brachystele* Schltr., whereas Garay (1982) maintained it as a separate, monospecific genus. On the other hand, Szlachetko (1991a) first merged *Microthelys* Garay with *Galeottiella*, but subsequently treated them as separate genera (Szlachetko, 1995; Szlachetko and Rutkowski, 2000).

Our results indicate that *Galeottiella sarcoglossa* is sister to the rest of the core spiranthids (Fig. 6) and should be excluded from Spiranthinae. The flowers have some features in common with Spiranthinae, such as being resupinate and bearing swollen nectar glands near the base of the labellum, but these characters are also present in Manniellinae and the latter also in some Prescottiinae (Dressler, 1993; Vargas, 1997). *Galeottiella* differs from Spiranthinae in that the labellum does not adhere to the sides of the column and the pollinia are homogeneous, i.e., the tetrads are not differentiated into a ''stalk'' portion and a fertile portion (see later). Other differences are discussed in Salazar et al. (2002a) and Salazar (in press). Salazar et al. (2002a) proposed a new subtribe, Galeottiellinae, to correspond with the phylogenetic position of *Galeottiella*.

Manniellinae—When describing *Manniella*, Reichenbach (1881) suggested a relation to *Stenoptera*, and Schlechter (1911) included it in Cranichidinae, although he expressed doubts about its relationships because of the inadequate material available to him. Subsequently Schlechter (1926) segregated *Manniella gustavi*, the only species of the genus known at that time, in a subtribe on its own, i.e., Manniellinae. A few additional species have been erroneously assigned to *Manniella*, but recently a second species genuinely belonging in this genus was discovered (Salazar et al., 2002b). Although some authors have included *Manniella* in Spiranthinae (Mansfeld, 1937; Dressler and Dodson, 1960; Brieger, 1974–1975; Dressler, 1974, Garay, 1982), most subsequent classifications have maintained Manniellinae as a distinct subtribe by virtue of the peculiar column morphology (Dressler, 1981, 1993; Burns-Balogh and Funk, 1986a, b; Szlachetko, 1995). Dressler (1981, 1993) considered *Manniella* to be closely related to *Pachyplectron* because of similarities in the plant and the flowers. Likewise, Szlachetko (1995) compared the large clinandrium of *Manniella* to that of *Pachyplectron* but disregarded a close relationship between them because of the distinctive labellar spur of the latter; instead, he considered *Manniella* to be closely related to his narrow concept of Spiranthinae.

Our phylogenetic analyses show that Manniellinae are sister to all core spiranthids except *Galeottiella* (Fig. 6). Their well-supported position as sister of the clade that includes subtribes Prescottiinae, Cranichidinae, and Spiranthinae justifies their current subtribal status. The column of *Manniella* is unusual in that, in newly open flowers, it is slightly reflexed and then strongly inflexed such that its apex is oriented in a plane transverse to the main axis of the column (see illustration in Hallé, 1965; also Dressler, 1981, 1993). The peculiar folding of the column has resulted in the anther of *Manniella* being interpreted as incumbent (Szlachetko and Rutkowski, 2000); however, in newly open flowers, it is the *whole column apex* that is inflexed, and therefore the anther is actually erect, i.e., parallel to the plane of the rostellum/stigma. As the flowers get older, the column gradually becomes nearly straight and thus indistinct from that of other Cranichideae. Such positional change of the column apex is directly related to protandry exhibited by *Manniella*, which has been described and illustrated in Salazar et al. (2002b).

Vargas (1997) viewed the presence of ''spiranthoid glands'' (nectar-secreting processes near the base of the labellum) as a synapomorphy of a Spiranthinae-Prescottiinae-Manniellinae clade, but this feature is more widespread and may represent a synapomorphy for core spiranthids (cf. Fig. 8).

Prescottiinae and Cranichidinae—Our results support monophyly of Cranichidinae but recover Prescottiinae as paraphyletic to both Cranichidinae and Spiranthinae (Fig. 6). Dressler (1990, 1993) established Prescottiinae to include several genera previously placed in Cranichidinae but differing in

TABLE 4. Number of steps, CI, and RI for transitions (ts) and transversions (tv) for DNA regions included in this study (ACCTRAN optimization on the MPT of the combined analysis, Figs. 6–7).

	rbcL		*matK*		*trnK* intron		*trnL* intron		*trnL-trnF* spacer		ITS1		5.8S		ITS2	
	ts	tv	ts	tv	ts	tv	ts	tv	ts	tv	ts	tv	ts	tv	ts	tv
No. steps	243	92	736	636	98	159	281	407	254	308	677	355	55	21	599	317
CI	0.60	0.61	0.62	0.50	0.67	0.53	0.66	0.48	0.72	0.50	0.34	0.48	0.58	0.62	0.38	0.50
RI	0.75	0.73	0.69	0.65	0.69	0.71	0.65	0.63	0.76	0.64	0.55	0.67	0.85	0.33	0.52	0.68
ts/tv ratio	2.64		1.16		0.62		0.69		0.82		1.91		2.62		1.89	

velamen type and rostellum and pollinarium characteristics. According to Dressler (1990, 1993), Cranichidinae possess velamen of the *Calanthe* type (after Porembski and Barthlott, 1988), a long, pointed rostellum terminated in a hamular viscidium and brittle pollinia, whereas in Prescottiinae velamen is of the *Spiranthes* type, the short laminar rostellum lacks a hamulus, and the pollinia are soft. However, a definite hamulus is present in the prescottioid genus *Gomphichis* (A. Álvarez, New York Botanical Garden, personal communication, 2001; G. A. Salazar, personal observation); this feature is easily overlooked and deserves further exploration to confirm whether it is present in other Prescottiinae.

Two clades of Prescottiinae were recovered in our analyses. One of these is a strongly supported Andean group with *Stenoptera*, *Gomphichis*, *Porphyrostachys*, and *Aa* (D, Fig. 6), and the other is moderately supported *Prescottia*, with *P. tubulosa* sister to *P. plantaginea*-*P.* affinis *oligantha*. *Prescottia tubulosa* is unusual in that genus in having a compact basal rosette of leaves that are usually withered at flowering time and an involute, not calceolate labellum (cf. Vargas, 1997). Vargas (1997) proposed the transfer of *P. tubulosa* to *Porphyrostachys* because of similarities in the deciduous habit and perianth morphology (the change, proposed in his master's thesis, has never been validly published). However, our results indicate that *Prescottia tubulosa* is more closely related to other species of the genus than to *Porphyrostachys* and that similarities with the latter are likely to represent convergence.

González Tamayo (1996) suggested the transfer of the cranichid genus *Pterichis* to Prescottiinae because of differences (of degree) in floral morphology, but our results confirm its placement in Cranichidinae. Other genera considered by González Tamayo (1996) to be out of place in Cranichidinae, including *Fuertesiella* Schltr. and *Nothostele* Garay, were not sampled for this study, but from the morphological information available, they seem best placed in Cranichidinae (cf. Szlachetko and Rutkowski, 2000). *Nothostele* and *Pseudocranichis* Garay, the latter a new genus established for a species previously described in *Cranichis*, were both included in Spiranthinae by Garay (1982). However, *Nothostele* has nonresupinate flowers and the pointed rostellum with hamular viscidium characteristic of Cranichidinae. On the other hand, *Pseudocranichis* has nonresupinate flowers and in column and labellum structure it is more similar to some members of Prescottiinae, especially *Prescottia tubulosa* (G. A. Salazar, personal observation). Garay (1982) described the labellum of *Pseudocranichis* as being adherent to the sides of the column, a typical feature of Spiranthinae; however, in the material of *Pseudocranichis* we studied, the labellum is stuck to the receptive portion of the stigma instead of being in contact with the nonreceptive sides of the column to form a tunnel to the nectar chamber. We suspect that the condition observed in *Pseudocranichis* is an artefact of pressing (only herbarium specimens of this little-known genus have been available for study) because it is difficult to understand how the receptive stigmatic surface could be functional if it is ''glued'' to the labellum at anthesis, especially as there is no evidence of self-pollination in this genus.

Spiranthinae—With the exclusion of *Galeottiella* (see earlier), Spiranthinae are strongly supported as monophyletic by our data (Fig. 6). There are, however, few obvious morphological synapomorphies for this clade (Fig. 8). One of these is the adhesion of the margins of the labellum to the sides of the

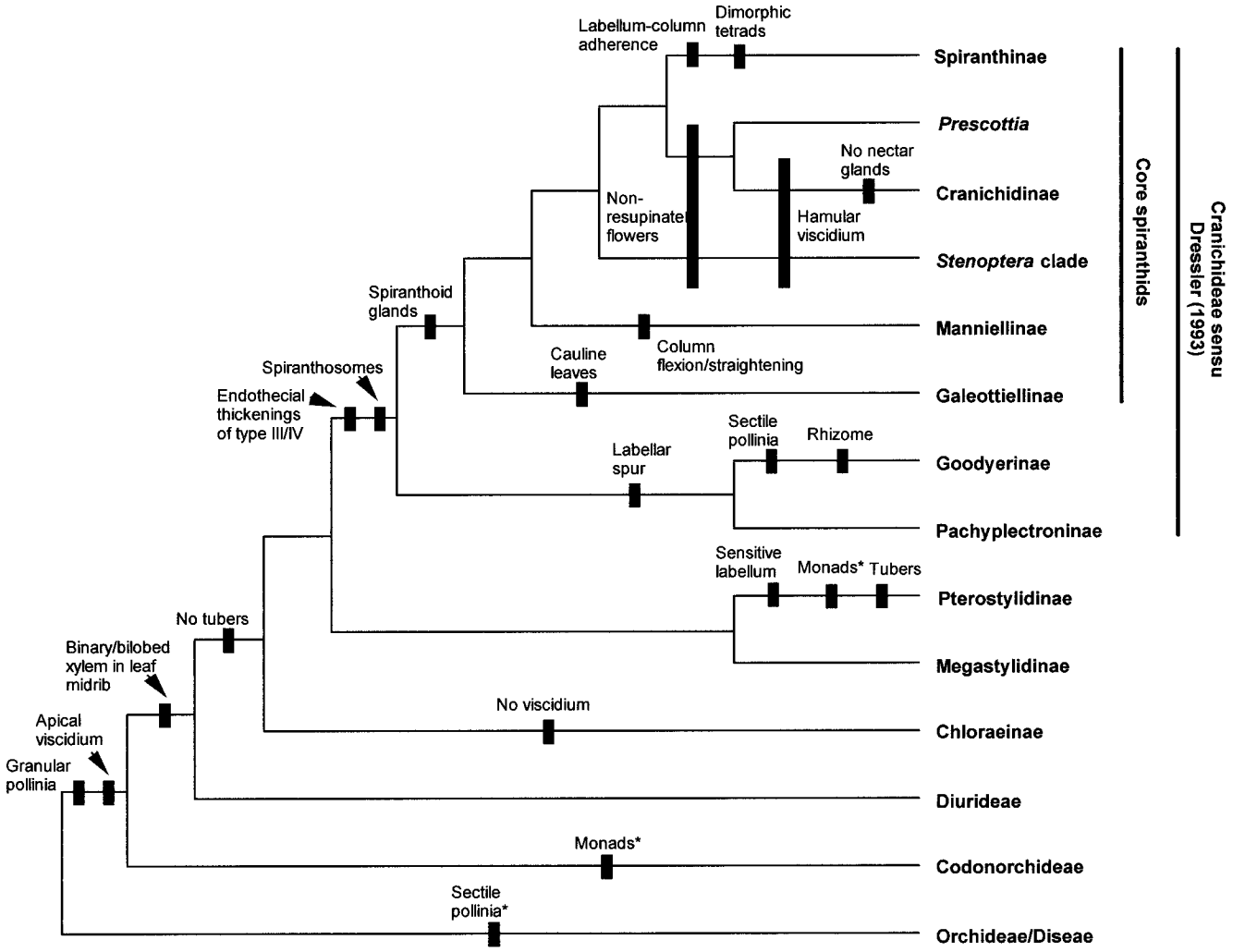

Fig. 8. Putative morphological synapomorphies for various clades identified in this study. The morphological characters were mapped on a simplified version of the most-parsimonious tree depicted in Fig. 6. An asterisk (*) indicates a character that appears more than once on the tree.

column to form a strong tunnel that leads to the nectar, which accumulates at or near the base of the perianth tube. To our knowledge, the only genus in Spiranthinae in which this character is absent is *Discyphus* Schltr., a little-known, monospecific South American genus with peculiar vegetative and floral morphology that has not been available for molecular study (Salazar, in press). A further putative morphological synapomorphy of Spiranthinae is a marked differentiation between the apical and basal tetrads of the pollinia. The apical tetrads are usually flattened and elongated, have comparatively thick, psilate or foveolate exine, and are densely packed and often oriented parallel to the longitudinal axis of the pollinia (like tiles on a roof) to form more or less definite pollinium stalks (Fig. 9A). In some cases, the pollen grains are arranged linearly rather than radially (Fig. 9C), or they form aggregations consisting of more than four grains and are best termed polyads (Fig. 9C–D). In contrast, basal tetrads are loosely arranged in a transverse plane to the longitudinal axis of the pollinium and are more or less rhomboid or oval in outline with the tetrads mostly arranged radially and usually reticulate or foveolate exine (Fig. 9B; see also Balogh, 1982 and Burns-Balogh, 1987, 1988). There is usually a gradual transition between these two extreme morphologies, blurring the distinction between pollinium body and stalk (although sometimes the pollinium stalks, or caudicles, are described as if they formed distinct structures; cf. Szlachetko and Rutkowski, 2000). There are some exceptions, such as the genera *Mesadenus* and *Microthelys*, in which the pollinia consist of loosely arranged, homogeneous tetrads, with the viscidium occupying a central (not apical) position on the ventral surface of the pollinarium. In Galeottiellinae, Manniellinae, and Prescottiinae, tetrads are homogeneous throughout the pollinia (Fig. 9E–G), whereas in Cranichidinae those forming the attenuate pollinium stalks are somewhat different in shape and arrangement from the basal tetrads (but apparently there are no differences in exine ornamentation; G. A. Salazar, unpublished data).

Several major clades within Spiranthinae were identified in this study that do not correspond with the limits of the generic alliances established by Balogh (1982; Burns-Balogh and Robinson, 1983; Burns-Balogh, 1986) or the narrowly defined subtribes into which Szlachetko (1995) subdivided Spiranthinae. A comparison of the subtribal limits of Szlachetko (1995) with the relationships found in this study (Fig. 7) shows that Spiranthinae sensu Szlachetko and Stenorrhynchidinae are polyphyletic, whereas Cyclopogoninae are paraphyletic. The major

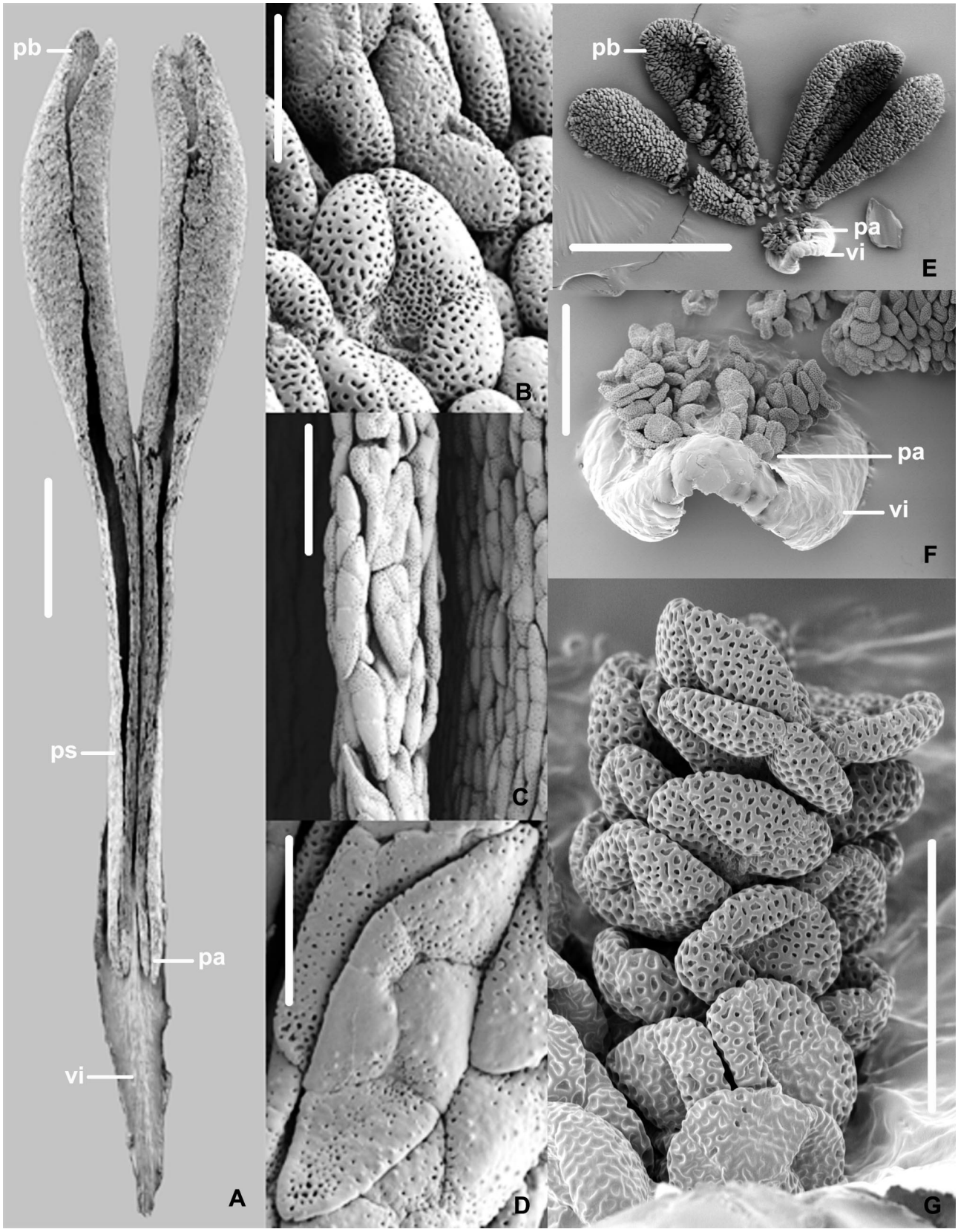

Fig. 9. Pollinarium characteristics of Spiranthinae and Prescottiinae. (A–D) *Sacoila lanceolata* (Aubl.) Garay (Spiranthinae), from *Salazar et al. 6226.* (A) Whole pollinarium (scale bar = 1 mm). (B) Tetrads from basal portion of pollinium (scale bar = 30 μm). (C) Tetrads/polyads from middle portion of pollinium stalk (scale bar = 100 μm). (D) Polyad from near pollinium apex (scale bar = 40 μm). (E–G) *Prescottia plantaginea* Lindl. (Prescottiinae), from *Salazar 6350.* (E) Whole pollinarium (scale bar = 500 μm). (F) Viscidium and apical portions of pollinia (scale bar = 100 μm). (G) Tetrads from apical portion of pollinium (scale bar = 40 μm). Figure abbreviations: pa, pollinium apex; pb, pollinium base; ps, pollinium stalk; vi, viscidium.

clades identified by our analyses are briefly discussed later. A more in-depth discussion of generic delimitation and relationships in Spiranthinae will be the focus of another contribution (G. A. Salazar and M. W. Chase, unpublished manuscript).

1. *Stenorrhynchos clade* (F, Fig. 7)—This group includes *Stenorrhynchos speciosum*, *Eltroplectris calcarata*, *Mesadenella petenensis*, and *Sacoila lanceolata*. Previously, *Stenorrhynchos* Rich. ex Spreng. has included species with a rigid, subulate rostellum (e.g., Lindley, 1840; Balogh, 1982; Garay, 1982; Burns-Balogh, 1986), although circumscription has varied greatly (cf. Garay, 1982 contra Balogh, 1982). *Coccineorchis* has a rigid, subulate rostellum and was treated as a section of *Stenorrhynchos* by Balogh (1982), but our data do not provide evidence for a relationship to the *Stenorrhynchos* lineage. Instead, *Coccineorchis cernua* obtained low support as sister to the *Pelexia* clade (G, Fig. 7). On the other hand, *Dichromanthus aurantiacus* has traditionally been considered a member of *Stenorrhynchos*, but our results indicate that it is not closely related to *S. speciosum*, the type species of the genus, instead belonging in a subclade of the well-supported *Spiranthes* clade (J, Fig. 7; see also Salazar et al., 2002a; Salazar, in press).

2. *Pelexia clade* (G, Fig. 7)—This clade includes *Cyclopogon epiphyticum*, *Sarcoglottis acaulis*, *Odontorrhynchus variabilis*, and *Pelexia adnata*. With the exception of *Odontorrhynchus*, all these genera have been grouped in the ''*Pelexia* alliance'' (Balogh, 1982; Burns-Balogh and Robinson, 1983) and the similarly delimited subtribe Cyclopogoninae (Szlachetko, 1995). Burns-Balogh and Robinson (1983) identified several putative synapomorphies for the *Pelexia* alliance, including an oblong, truncate, or shallowly notched rostellum, apical viscidium situated between the apices of the pollinium stalks and facing the anther, and the anther bearing an apical extension that covers the apex of the rostellum during flower development. *Odontorrhynchus*, on the other hand, was referred to the *Brachystele* alliance (Balogh, 1982: 121), which included as well the genus *Sauroglossum* Lindl. and was characterized by a ''reduced rostellum which, except for an apiculate or triangular process, is usually totally removed with the viscidium'' and a ventral viscidium that leaves a U-shaped notch at the apex of the column (Balogh, 1982; Burns-Balogh and Robinson, 1983). However, some species of *Pelexia*, such as *P. novofriburgensis* (Rchb. f.) Garay and *P. weberbaueri* (Kränzl.) Schltr., have a short rostellum and apical viscidium and are similar to the condition observed in various species of *Sauroglossum* (Salazar, in press). Furthermore, the anther in most species of *Brachystele*, *Odontorrhynchus*, and *Sauroglossum* has a distinct apical extension similar to that found in the *Pelexia* alliance (cf. Szlachetko and Rutkowski, 2000). A preliminary analysis of ITS and *trnL-F* sequences of a broader sample of Spiranthinae genera placed *Sauroglossum* as sister to the *Pelexia-Odontorrhynchus* clade, indicating paraphyly of the *Pelexia* alliance with respect to the *Brachystele* alliance (G. A. Salazar, M. W. Chase, and A. Álvarez, unpublished data). On the basis of rostellum and viscidium morphology, Singer and Coccuci (1999) suggested that *Odontorrhynchus* may represent an intermediate evolutionary stage between the *Brachystele* and *Stenorrhynchos* alliances (sensu Balogh, 1982), but such an idea is not supported by our data and is difficult to evaluate without a morphological cladistic analysis.

3. *Spiranthes clade* (H–J, Fig. 7)—This group includes *Spiranthes* Rich. (sensu stricto) and several other genera dispersed among Balogh's (1982) *Spiranthes* and *Stenorrhynchos* alliances and, more recently, among subtribes Stenorrhynchidinae and Spiranthinae sensu Szlachetko (1995). One of the two main subclades in this group (H) includes *Svenkoeltzia congestiflora*, *Beloglottis costaricensis*, and *Aulosepalum tenuiflorum*, which are collectively sister to *Microthelys* affinis *minutiflora*, *Funkiella hyemalis*, and *Schiedeella faucisanguinea*. Both *Schiedeella faucisanguinea* and *Microthelys* differ in flower size and rostellum structure from *Funkiella*, but share with it the thickened, showily colored areas on the labellum and are found in similar habitats (high montane vegetation, often above 3000 m). The other subclade (I–J) includes *Spiranthes* sister to a clade with *Mesadenus lucayanus*, *Schiedeella llaveana*, *Deiregyne diaphana*, *Dichromanthus aurantiacus*, and *D. cinnabarinus*. *Schiedeella*, typified by *S. llaveana*, as currently delimited is polyphyletic (e.g., Szlachetko, 1992).

4. *Eurystyles*—This distinctive genus is weakly supported as sister to the *Spiranthes* clade. Balogh (1982) and Burns-Balogh et al. (1985) considered *Eurystyles* to form a distinct alliance characterized by the epiphytic plants with rosulate leaves, capitate inflorescence, bracts with ciliated margins, and nonresupinate flowers. Although our present analysis included a single representative of this group, ongoing phylogenetic study of Spiranthinae based on ITS, *trnL-F*, and *matK* sequences of a larger sample of both genera and species indicates that *Eurystyles* (including *Synanthes* Burns-Bal., H. Rob. & M. S. Foster and *Pseudoëurystyles* Hoehne) is monophyletic and most closely related to *Lankesterella* Ames (G. A. Salazar and M. W. Chase, unpublished manuscript). This relationship was suggested previously by Dressler (1981) and Soto Arenas (1993), in spite of differences in floral structure, on the basis of their uniquely shared habit, i.e., obligatorily epiphytic evergreen rosettes of minute, varnished leaves usually with ciliate margins. In contrast, Balogh (1982) reduced *Lankesterella* to a section of *Stenorrhynchos*, whereas Szlachetko (1995) placed *Eurystyles* in his version of Spiranthinae and *Lankesterella* in Stenorrhynchidinae.

General conclusions—This study is the first molecular phylogenetic assessment of tribe Cranichideae and has permitted us to evaluate their monophyly and subtribal classification from a perspective independent from the morphological criteria used originally to delimit the taxa. Dressler's (1993) circumscription of Cranichideae corresponds to a clade, as do subtribes Goodyerinae, Cranichidinae, and Spiranthinae once *Galeottiella* is excluded (as proposed in Salazar et al., 2002a). Prescottiinae, on the other hand, seem to represent a grade with two clades (*Prescottia* and *Stenoptera* through *Aa*) for which relationships to Cranichidinae and Spiranthinae are not clearly supported. This lack of support indicates the need to reevaluate these portions of the evolutionary tree of Cranichideae using a broader sample of both characters and taxa to attain conclusive ideas about their relationships. On the other hand, the important discrepancies between well-supported monophyletic groups of Spiranthinae genera found in this study and current generic and supra-generic classifications highlight the need to re-examine the morphological characters (mostly attributes of the rostellum and viscidium) on which such classifications have been based. These and other floral

characters used for taxonomic delimitation in Spiranthinae are directly involved in pollination, and thus prone to homoplasy resulting from pressures from pollinators (cf. Chase and Palmer, 1997; Hapeman and Inoue, 1997; Pridgeon et al., 2001). Strongly supported relationships inferred from the molecular data analyzed here provide a foundation for further phylogenetic studies focused on both improving classification and constructing an unbiased, independent framework for studying the evolution of floral morphology, pollination, and other biological traits.

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