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Phylogeny of South American Pogonieae (Orchidaceae, Vanilloideae) based on sequences of nuclear ribosomal (ITS) and chloroplast (*psaB*, *rbcL*, *rps16*, and *trnL-F*) DNA, with emphasis on *Cleistes* and discussion of biogeographic implications

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Abstract

Tribe Pogonieae (Orchidaceae), as currently known, comprises five genera distributed from South to North America and Eastern Asia. Phylogenetic inferences within *Cleistes* and among genera of tribe Pogonieae were made based on nrDNA (ITS) and cpDNA (*trnL-F*, *rps16*, *rbcL*, and *psaB*) sequence data and maximum parsimony. Eighteen species of *Cleistes*, members of all other genera of Pogonieae, and outgroups were sampled. Analyses based on individual DNA regions provided similar topologies. All evidence indicates that *Cleistes* is paraphyletic. The North American *C. divaricata* and *C. bifaria* are more closely related to the temperate genera *Isotria* and *Pogonia* than to their Central and South American congeners, the latter constituting a monophyletic group characterized by the production of nectar as reward, tuberous roots, and their distribution in Central and South America. The Amazonian *Duckeella* is sister to the remainder of Pogonieae. Taxonomic and biogeographic implications are discussed, and morphological synapomorphies are given for clades obtained in the inferred molecular phylogeny.

Keywords: Orchidaceae, Vanilloideae; Pogonieae; ITS; *rbcL*; *trnL-F*; *rps16*; *psaB*

Introduction

Tribe Pogonieae (i.e. subtribe Pogoniinae; Dressler 1993; Cameron et al. 1999; Cameron and Chase 1999) includes five genera distributed from South to North America and Eastern Asia (Cameron 2003). The largest genus of the tribe is *Cleistes*, with about 20 species (Pansarin and Barros, unpublished). The remaining genera, namely *Duckeella*, *Isotria*, *Pogonia*, and *Pogoniopsis*, are much smaller or even monotypic (Cameron 1999). The suite of morphological characters that defines Pogonieae and other vanilloid orchids makes these taxa important to orchid systematics, because these characteristics occur nowhere else in Orchidaceae and may provide clues to the evolution of this large and diverse family (Cameron and Chase 1999). In fact, these orchids have a considerable number of plesiomorphic

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features that have led orchidologists to classify them at various levels and to disagree about their circumscription and phylogenetic position within Orchidaceae (Cameron and Chase 1999).

The position of vanilloid orchids in Orchidaceae has been controversial, so that various taxonomic ranks within the family have been assigned to them (see Cameron and Chase 1999 for a review).

In recent treatments based on morphological and DNA sequence data (Cameron et al. 1999; Cameron and Chase 1999; Chase et al. 2003; Cameron 2004; Freudenstein et al. 2004), and in accordance with Szlachetko (1995), the vanilloid orchids were ranked at subfamily level. Basically, the group corresponds to tribe Vanilleae sensu Dressler (1993), but including Pogonieae. Subtribes Vanillinae, Galeolinae and Pogoniinae were recognized as taxa within subfamily Vanilloideae (Cameron et al. 1999). In his most recent treatment of the vanilloid orchids, Cameron (2003) elevated subtribe Pogoniinae to tribal rank (Pogonieae), and combined Galeolinae and Vanillinae to tribe Vanilleae. According to Cameron et al. (1999) and Cameron and Chase (1999), based on morphology and DNA sequence data of the nuclear ribosomal internal transcribed spacers (ITS) and plastid *rbcL*, tribe Pogonieae is monophyletic. The South American genus *Duckeella* is sister to all remaining genera of Pogonieae, and *Cleistes* is paraphyletic (Cameron and Chase 1999). The North American *C. divaricata* and *C. bifaria* are more closely related to the North American *Isotria* and the North American–Asiatic *Pogonia* than to their South American congeners; the South American *Cleistes* constitute a monophyletic group (Cameron and Chase 1999). The rare saprophytic *Pogoniopsis* has not been included in these analyses; thus its position within tribe Pogonieae remains inconclusive (Cameron and Chase 1999; Cameron 2003).

DNA sequence data have been used to infer the phylogeny of Orchidaceae (e.g. Cameron et al. 1999; Cameron 2004; Freudenstein et al. 2004). The nrDNA ITS and the plastid DNA regions *rps16* and *trnL-F* have been useful for the reconstruction of phylogenetic relationships at low hierarchic levels in Orchidaceae (e.g. Cameron and Chase 1999; Clements et al. 2002; Koehler et al. 2002; Bateman et al. 2003; Smith et al. 2004); *psaB* and *rbcL* have been useful concerning relationships among higher taxa within the family (e.g. Cameron et al. 1999; Cameron 2004).

The present paper aims at (1) forming a hypothesis about phylogenetic relationships among the species currently recognized as *Cleistes*, (2) reconstructing phylogenetic and biogeographic relationships among South American Pogonieae, (3) indicating synapomorphic characters delimiting taxa within subtribes, and (4) suggesting taxonomic changes coherent with the obtained phylogeny.

Material and methods

Taxon sampling

A total of twenty-seven species were sampled, representing all five genera (*Cleistes*, *Duckeella*, *Isotria*, *Pogonia*, and *Pogoniopsis*) currently recognized as Pogonieae and here treated as the ingroup. Species of *Vanilla* were used as outgroups; in addition, *Epistephium* (also a vanilloid genus) was included in some analyses based on previous morphological and molecular phylogenetic studies of Orchidaceae (Cameron et al. 1999; Cameron 2004; Freudenstein et al. 2004), particularly of tribe Pogonieae (Cameron and Chase 1999). The ingroup and outgroup species used in this study, voucher information and GenBank accession numbers are given in Table 1.

DNA extraction, amplification and sequencing

Total DNA was extracted from fresh and silica gel-dried plant issues, following a modified CTAB method of Doyle and Doyle (1987). The product obtained was analyzed by electrophoresis using 1% agarose gel and ethidium bromide to test DNA quality and relative quantity. Amplifications were carried out using 50 µl PCR reaction volumes and *Taq* DNA polymerase. Primers for amplification of ITS1, ITS2 and intervening 5.8 S (Sun et al. 1994), plastid *trnL* intron and *trnL-F* intergenic spacer (Taberlet et al. 1991), *rps16* intron (Oxelman et al. 1997), *rbcL* (Chase et al. 1994) and *psaB* (Cameron 2004) were used for reactions for amplification and sequencing. For ITS amplification, 1.0 ml/l betaine (Sigma) was added to the PCR mixture. *Taq* DNA polymerase was added to the mixture in the thermocycler at 80 °C, after a period of 10 min of denaturation at 99 °C; 35 cycles were then carried out according to a program of denaturation for 1 min at 94 °C, annealing for 45 s at 65 °C, extension for 1 min at 72 °C, and final extension for 5 min at 72 °C. For *trnL-F* the program was denaturation for 2 min at 94 °C, then 33 cycles of denaturation for 1 min at 94 °C, annealing for 45 s at 56 °C, extension for 80 s at 72 °C, final extension for 5 min at 72 °C. For *rps16*, *rbcL* and *psaB* the thermocycler program was similar to that for *trnL-F*, with annealing at 50 °C (*rps16* and *rbcL*) or 60 °C (*psaB*). Successfully amplified PCR products were cleaned using GFX PCR purification kits (Amersham Biosciences). Sequences were obtained with Applied Biosystems automated sequencer models 3100 or 3700, using Big Dye 3.0–3.1 (ABI) following the manufacturer's protocols.

For sequence editing and assembly of complementary and overlapping sequences, the ABI softwares Sequence Navigator and Autoassembler (Applied Biosystems),

Table 1. Species of Pogonieae and Vanilleae included in the molecular study, data collected, and GenBank accession numbers

Species	Voucher	Data collected	GenBank accessions
Cleistes aphylla (Barb. Rodr.) Hoehne	Pansarin and Mickeliunas 899 (UEC)	ITS, *trnL-F*	EU498138, EU498194
Cleistes aphylla (Barb. Rodr.) Hoehne	Pansarin and Mickeliunas 933 (UEC)	*rps16*	EU498166
Cleistes aphylla (Barb. Rodr.) Hoehne	Mickeliunas and Pansarin 14 (UEC)	ITS, *rps16*, *rbcL*, *psaB*	EU498139, EU498167, EU498117, EU498094
Cleistes bella Rchb. f. & Warm.	Pansarin and Mickeliunas 921 (UEC)	*trnL-F*	EU498195
Cleistes bella Rchb. f. & Warm.	Mickeliunas and Pansarin s.n. (UEC)	ITS, *rps16*, *rbcL*, *psaB*	EU498140, EU498168, EU498118, EU498095
Cleistes bifaria (Fernald) Catling & Gregg	Rothacker 60 (UEC)	ITS, *rps16*, *trnL-F*, *rbcL*, *psaB*	EU498141, EU498169, EU498224, EU498119, EU498098
Cleistes caloptera (Rchb. f. & Warm.) Schltr.	Pansarin and Mickeliunas 1172	*rbcL*	EU498120
Cleistes castanoides Hoehne	Pansarin and Mickeliunas 873 (UEC)	*rps16*	EU498170
Cleistes castanoides Hoehne	Pansarin and Mickeliunas 932 (UEC)	*trnL-F*, *rbcL*, *psaB*	EU498197, EU498121, EU498096
Cleistes castanoides Hoehne	Pansarin et al. s.n. (UEC)	*trnL-F*	EU498196
Cleistes cipoana Hoehne	Pansarin and Mickeliunas 915 (UEC)	ITS, *rps16*, *trnL-F*, *rbcL*, *psaB*	EU498142, EU498171, EU498198, EU498133, EU498097
Cleistes exilis Hoehne	Pansarin and Mickeliunas 898 (UEC)	ITS, *trnL-F*	EU498144, EU498201
Cleistes exilis Hoehne	Pansarin and Mickeliunas 923 (UEC)	*trnL-F*, *psaB*	EU498200, EU498099
Cleistes exilis Hoehne	Mickeliunas and Pansarin 40 (UEC)	ITS, *rps16*, *rbcL*, *psaB*	EU498145, EU498173, EU498122, EU498100
Cleistes exilis Hoehne	Pansarin and Simões 783 (UEC)	ITS, *rps16*, *trnL-F*	EU498143, EU498172, EU498199
Cleistes divaricata (L.) Ames	Goldman and Brzuszek 2287 (UEC)	ITS, *rbcL*, *psaB*	AF151009 [a], AF074127 [a], AY380957 [a]
Cleistes gracilis (Barb. Rodr.) Schltr.	Pansarin and Mickeliunas 908 (UEC)	*trnL-F*, *rbcL*, *psaB*	EU498203, EU498123, EU498101
Cleistes gracilis (Barb. Rodr.) Schltr.	Pansarin and Mickeliunas 1123 (UEC)	ITS, *rps16*	EU498147, EU498175
Cleistes gracilis (Barb. Rodr.) Schltr.	Mickeliunas and Pansarin 46 (UEC)	ITS, *rps16*, *trnL-F*, *rbcL*, *psaB*	EU498146, EU498174, EU498202, EU498124, EU498102
Cleistes itatiaiae Pabst	Pansarin and Mickeliunas 1019 (UEC)	ITS, *rps16*, *trnL-F*	EU498148, EU498176, EU498205
Cleistes itatiaiae Pabst	Pansarin and Mickeliunas 1007 (UEC)	*trnL-F*	EU498204
Cleistes itatiaiae Pabst	Pansarin and Mickeliunas 1129 (UEC)	ITS, *rps16*, *rbcL*, *psaB*	EU498149, EU498177, EU498125, EU498104
Cleistes libonii (Rchb. f.) Schltr.	Pansarin 857 (UEC)	*rps16*, *trnL-F*	EU498179, EU498206
Cleistes libonii (Rchb. f.) Schltr.	Pansarin and Mickeliunas 972 (UEC)	ITS, *rps16*, *trnL-F*, *rbcL*, *psaB*	EU498150, EU498178, EU498207, EU498126, EU498114
Cleistes metallina (Barb. Rodr.) Schltr.	Pansarin et al. 776 (UEC)	*trnL-F*	EU498208
Cleistes metallina (Barb. Rodr.) Schltr.	Pansarin and Mickeliunas 909 (UEC)	ITS	EU498151
Cleistes metallina (Barb. Rodr.) Schltr.	Pansarin and Mickeliunas 1119 (UEC)	*rbcL*, *psaB*	EU498127, EU498105
Cleistes metallina (Barb. Rodr.) Schltr.	Pansarin and Mickeliunas 1145 (UEC)	*rps16*, *trnL-F*	EU498180, EU498209
Cleistes moritzii (Rchb. f.) Garay & Dunsterv.	Pansarin et al. 782 (UEC)	*rps16*, *trnL-F*, *psaB*	EU498183, EU498212, EU498108
Cleistes paranaensis (Barb. Rodr.) Schltr.	Pansarin and Mickeliunas 920 (UEC)	*rps16*, *trnL-F*	EU498182, EU498210
Cleistes paranaensis (Barb. Rodr.) Schltr.	Mickeliunas and Pansarin 31 (UEC)	ITS, *rbcL*, *psaB*	EU498152, EU498128, EU498106
Cleistes paranaensis (Barb. Rodr.) Schltr.	Pansarin and Batista 759 (UEC)	*rps16*	EU498181
Cleistes paranaensis (Barb. Rodr.) Schltr.	Simões et al. s.n. (UEC)	*trnL-F*	EU498211

Table 1. (*continued*)

Species	Voucher	Data collected	GenBank accessions
Cleistes pusilla Pansarin	Pansarin and Mickeliunas 901 (UEC)	*rps16*	EU498184
Cleistes pusilla Pansarin	Pansarin and Mickeliunas 922 (UEC)	ITS, *trnL-F*	EU498153, EU498213
Cleistes pusilla Pansarin	Mickeliunas and Pansarin 12 (UEC)	*rbcL*, *psaB*	EU498129, EU498107
Cleistes ramboi Pabst	Pansarin and Mickeliunas s.n. (UEC)	ITS, *trnL-F*, *rbcL*	EU498154, EU498214, EU498130
Cleistes aff. *ramboi* Pabst	Pansarin et al. s.n. (UEC)	*rps16*, *trnL-F*, *psaB*	EU498185, EU498215, EU498109
Cleistes rosea Lindl.	K. Cameron 1038 (NCU)	*rbcL*, *psaB*	AF074128 [a], AY380958 [a]
Cleistes tenuis Rchb. f.	Mickeliunas and Pansarin 13 (UEC)	ITS, *rps16*, *trnL-F*	EU498155, EU498187, EU498217
Cleistes tenuis Rchb. f.	Pansarin and Mickeliunas 940 (UEC)	ITS, *rps16*, *trnL-F*, *rbcL*, *psaB*	EU498156, EU498186, EU498216, EU498131, EU498103
Cleistes uliginosa Pabst	Pansarin and Mickeliunas 872 (UEC)	ITS, *trnL-F*	EU498157, EU498218
Cleistes uliginosa Pabst	Pansarin and Mickeliunas 937 (UEC)	*rps16*	EU498188
Cleistes uliginosa Pabst	Pansarin and Mickeliunas 984 (UEC)	ITS, *rps16*, *rbcL*, *psaB*	EU498158, EU498189, EU498132, EU498110
Duckeella adolphii Porto & Brade	Pansarin 1165 (INPA)	ITS, *rps16*, *trnL-F*, *rbcL*, *psaB*	EU498159, EU498190, EU498219, EU498134, EU498112
Epistephium lucidum Cogn.	M. Chase O-795 (K)	*rbcL*, *psaB*	AF074161 [a], AY381001 [a]
Epistephium parviflorum Lindl.	M. Chase O-794 (K)	*psaB*	AY381002 [a]
Epistephium sclerophyllum Lindl.	Mickeliunas and Pansarin 4 (UEC)	*trnL-F*	EU498221
Epistephium sclerophyllum Lindl.	Pansarin and Mickeliunas 953 (UEC)	*trnL-F*, *rbcL*	EU498220, EU498137
Isotria verticillata (Muhl. *ex* Willd.) Raf.	Rothacker 69 (UEC)	ITS, *rps16*, *trnL-F*, *rbcL*, *psaB*	EU498160, EU498191, EU498226, EU498135, EU498111
Isotria medeoloides Rafin.	P. Keenan s.n.	*rbcL*, *psaB*	AY381123 [a], AY381022 [a]
Pogonia japonica Rchb. f.	Cameron 1034 (NY)	ITS, *rbcL*, *psaB*	AF151011 [a], AF074219 [a], AY381061 [a]
Pogonia ophioglossoides (L.) Jussieu	Rothacker 70 (UEC)	ITS, *rps16*, *trnL-F*, *rbcL*, *psaB*	EU498161, EU498192, EU498225, EU498136, EU498113
Pogonia minor (Makino) Makino	Cameron 1033 (NY)	ITS, *rbcL*, *psaB*	AF151010 [a], AF074220 [a], AY381062 [a]
Pogoniopsis nidus-avis Rchb. f.	Mickeliunas and Pansarin 45 (UEC)	ITS	EU498162
Vanilla bahiana Hoehne	Pansarin 727 (UEC)	ITS, *rbcL*	EU498163, EU498115
Vanilla edwallii Hoehne	Pansarin 840 (UEC)	ITS, *rps16*, *trnL-F*, *rbcL*, *psaB*	EU498165, EU498193, EU498222, EU498116, EU498093
Vanilla palmarum (Salzm. *ex* Lindl.) Lindl.	Pansarin 1168 (INPA)	*psaB*	EU498092
Vanilla pompona Schiede	Pansarin 1167 (INPA)	ITS, *trnL-F*	EU498164, EU498223

[a]Sequences taken from GenBank.

respectively, were used. DNA sequences were aligned using Clustal X version 1.83 (Thompson et al. 1997). Subsequent manual corrections were carried out with BioEdit version 5.0.9. Indels were entered as missing data and added to the sequence data as binary characters for phylogenetic analyses. Matrix ends were trimmed to exclude sequence artifacts. Taxa used in the analyses and GenBank accessions are listed in Table 1. Matrices are available from the corresponding author upon request.

Data matrix composition and cladistic analysis

All cladistic analyses were run with PAUP* version 4.0b5 (Swofford 2001), using Fitch parsimony (Fitch 1971), including autapomorphies, and ACCTRAN optimization with zero-length branches collapsed. Heuristic searches were conducted with 28 taxa for *psaB*, 29 for *rbcL*, 23 for *trnL-F* and ITS (comprising ITS1, 5.8S and ITS2), 20 for *rps16*, and 18 for the five DNA regions combined. The search strategy for individual and combined data used 1000 replicates of random taxon entry additions, option MULTREES, and tree bisection-reconnection (TBR) swapping, holding 10 trees per replicate and saving all shortest trees. Support for clades was assessed using 1000 bootstrap replicates (Felsenstein 1985). In order to reduce the effect of homoplastic characters on the tree topologies, successive weighting (Farris 1969) was applied to the combined molecular data sets. The Partition Homogeneity Test of PAUP* 4.0b5 (Swofford 2001) was used to assess congruency among molecular data phylogenies. This test is equivalent to the ILD test of Farris et al. (1994) which has been employed for determining whether different data sets can be combined in one parsimony analysis. The test was conducted using parsimony and the following parameters: heuristic search, TBR branch-swapping, with 100 random addition sequences, and 500 replicates to generate the null hypothesis. Evolutionary trends of morphological character states were traced on the combined molecular trees with MacClade version 4.0 (Maddison and Maddison 2000).

Non-molecular characters

Specimens collected in the field and from the herbaria CEN, ESA, HB, HRCB, HUEFS, INPA, MBML, R, RB, SP, SPF, UEC, VEN, and VIC were used as sources of morphological characters. Voucher specimens were deposited in the UEC and INPA herbaria (Table 1). Characters were also obtained from papers by Thien and Marcks (1972), Mehrhoff (1983), Gregg (1989), Dressler (1993), Cameron and Chase (1998, 1999), and Cameron (2003).

Scanning electron microscopy (SEM) of *Pogoniopsis nidus-avis* seeds was carried out using mature fruits collected in the field. Seeds were mounted on aluminum SEM stubs and directly sputter-coated with gold–palladium alloy. Photographs were taken at an accelerating voltage of 10 kV. Seed characters of other taxa were obtained from Cameron and Chase (1998) and Dressler (1993).

Results

Information about tree statistics for the individual and combined data sets is given in Table 2, including tree lengths, consistency and retention indexes (CI and RI, respectively) and numbers of characters in the data matrices, variable characters, potentially phylogenetically informative characters, and most parsimonious trees. The ITS analysis gave the best results in terms of numbers of either variable or phylogenetically informative characters (514 and 399, respectively; Table 2). The number of most parsimonious trees obtained from the *psaB* analysis (4) was the lowest, compared to 6 obtained from both ITS and *rbcL*, and 30 obtained from both *rps16* and *trnL-F* analyses (Table 2). The ITS, *rps16* and *rbcL* analyses yielded consensus trees with relatively good resolution and strong support for many of the internal nodes. On the other hand, *trnL-F* and *psaB* analyses resulted in consensus trees with many polytomies. The results of the partition homogeneity test suggested that the five molecular data sets were

Table 2. Summary of results of the phylogenetic analyses of Pogonieae (Orchidaceae)

Parameter	ITS	*rps16*	*trnL-F*	*rbcL*	*psaB*	Molecular combined
Characters in matrix	768	936	478	1308	1653	5136
Variable characters	514 (66.9%)	585 (62.5%)	201 (42%)	225 (17.2%)	352 (21.3%)	1569 (30.5%)
Phylogenetically informative characters	399 (51.9%)	244 (26%)	145 (30.3%)	129 (9.9%)	188 (11.3%)	666 (12.7%)
Most parsimonious trees	6	30	30	6	4	2
Steps	1285	982	345	319	516	2563
Consistency index	0.71	0.79	0.80	0.79	0.77	0.81
Retention index	0.79	0.71	0.86	0.86	0.80	0.68

congruent. Phylogenetic analysis of the combined ITS, *rps16*, *trnL-F*, *rbcL* and *psaB* sequences resulted in 2 most parsimonious trees, with 1569 variable and 666 informative characters, of 2563 steps length, and with CI 0.81 and RI 0.68 (Table 2). The consensus tree is nearly completely resolved; most internal nodes are robustly supported (Fig. 1).

Attempts to amplify cpDNA regions of the saprophytic genus *Pogoniopsis* were unsuccessful; thus no phylogenetic analyses involving *rps16*, *trnL-F*, *rbcL* and *psaB* were conducted including this taxon. On the other hand, attempts to amplify ITS1, ITS2 and 5.8 S failed for *Cleistes rosea*, *C. castanoides* and *C. moritzii*; thus the phylogenetic analyses involving these species were restricted to cpDNA sequences.

Phylogenetic analyses of isolated ITS, *rps16*, *trnL-F*, *rbcL* and *psaB* regions

Strict consensus trees with strong internal support were obtained from individual ITS, *rbcL* and *rps16* phylogenetic analyses, not from any *psaB* and *trnL-F* analyses, but all trees have similar topologies. The vanilloid genus *Epistephium*, included in the *trnL-F*, *psaB* and *rbcL* analyses, emerged as sister to Pogonieae (Fig. 1). In the ITS consensus tree, *Pogoniopsis* is sister to all genera currently recognized as Pogonieae, which form a clade with bootstrap support (BS) 96. In the consensus trees of the ITS, *trnL-F*, *rbcL* and *psaB* analyses the Amazonian *Duckeella* is basal in the Pogonieae clade; in the *rps16* tree, this genus is part of

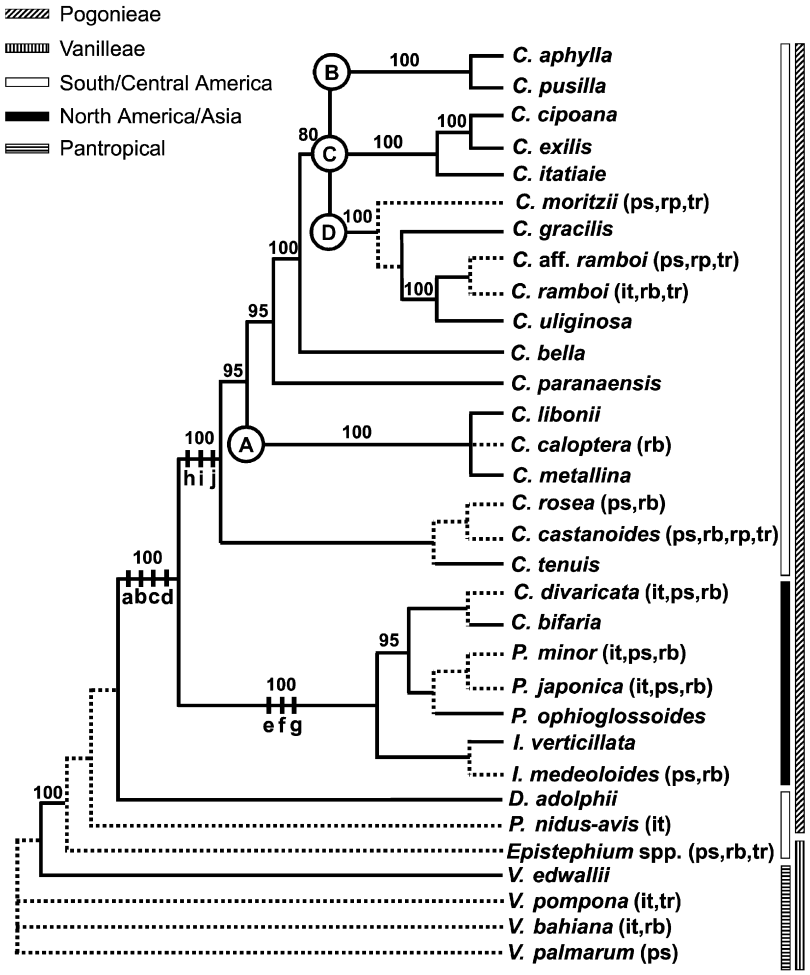

Fig. 1. Bootstrap consensus tree from combined molecular data of Pogonieae (Orchidaceae). Bootstrap values >50 are given above branches. Broken lines refer to taxa for which less than the complete set of marker DNA sequences was obtained. Lower-case letters (a–j) below branches refer to morphological synapomorphies diagnostic for Pogonieae, some of them (e–g) of temperate, others (h–j) of tropical taxa, as follows: a = fibrous roots; b = stem center hollow; c = inflorescence terminal; d = few flowers with white, pink or violet perianth parts; e = chromosome number $2n = 18$; f = epidermal cells sinuous; g = North American–Eastern Asian distribution; h = roots tuberous; i = nectar production; j = Central- and/or South American distribution. Capital letters (A–D) denote clades discussed in the text. For full scientific names of taxa see Table 1. Abbreviations: it = ITS; ps = *psaB*; rb = *rbcL*; rp = *rps16*; tr = *trnL-F*.

a polytomy at the clade base. The North American *Isotria*, *Cleistes divaricata* and *C. bifaria*, and the North American–Asiatic *Pogonia* compose a monophyletic temperate group (Fig. 1), in some analyses with high BS support. In the ITS, *rbcL* and *psaB* analyses, the North American *Pogonia ophioglossoides* emerges at the base of a clade with robust support, which contains a subclade formed by the Asian *P. japonica* and *P. minor* (Fig. 1). The clade that includes *C. divaricata* and *C. bifaria* is sister to *Isotria* and *Pogonia* in the ITS analysis. In the same analysis, the temperate clade combining *C. bifaria*, *C. divaricata*, *Isotria* and *Pogonia* is sister to the strongly supported (BS 98) South and Central American *Cleistes* clade. Similar results were obtained in the *rps16*, *trnL-F*, *rbcL* and *psaB* analyses. The North American *C. bifaria* and *C. divaricata* are more akin to the Asian *Isotria* and *Pogonia* than to their South and Central American congeners. According to the present results, *Cleistes* as currently circumscribed is paraphyletic. Regarding the Central and South American *Cleistes*, the ITS and *rps16* analyses yielded clades with robust support, such as (*C. aphylla* + *C. pusilla*) (BS 98 and 100, respectively), (*C. libonii* + *C. metallina*) (BS 98 and 99, respectively), (*C. gracilis* (*C. ramboi* + *C. uliginosa*)) (BS 100 and 88, respectively), (*C. exilis* + *C. cipoana*) (BS 98 and 76, respectively). In the *trnL-F* tree the tropical clade of *Cleistes* is poorly resolved, with internal clades weakly supported. Nonetheless, some consistent internal clades in the ITS, *rps16*, *rbcL* and *psaB* analyses are also apparent in the *trnL-F* tree, such as (*C. aphylla* + *C. pusilla*), (*C. gracilis* + *C. uliginosa*) and (*C. libonii* + *C. metallina*). All five individual phylogenetic analyses assign a basal position in the *Cleistes* tropical clade to either *C. tenuis* or the clade (*C. tenuis* + *C. rosea*) or (*C. tenuis* (*C. castanoides* + *C. rosea*)) (Fig. 1). The clade (*C. metallina* + *C. libonii*) is sister to a strongly supported clade containing *C. aphylla*, *C. bella*, *C. cipoana*, *C. exilis*, *C. gracilis*, *C. itatiaiae*, *C. paranaensis*, *C. pusilla*, *C. ramboi*, and *C. uliginosa*, with BS 99 and 98 in the ITS and *rps16* phylogenetic analyses, respectively. In the *rps16* phylogenetic tree, *C. itatiaiae* groups with *C. exilis* and *C. cipoana* with BS 91.

Analysis of combined molecular data

Analysis of combined data from the five DNA regions resulted in two most parsimonious trees (Fig. 1) with a topology largely congruent with those from the individual analyses. For example, as in the individual molecular analyses, the Amazonian *Duckeella* is sister to the remainder of Pogonieae, forming a monophyletic group with BS 100 (Fig. 1). *Isotria*, *Pogonia* and *Cleistes bifaria* together form a North American–Asiatic clade with BS 100, which in turn is sister to the Central and South American *Cleistes*. Thus, *Cleistes* is a paraphyletic genus. The North American *C. bifaria* has closer affinities to the temperate *Isotria* and *Pogonia* than to tropical *Cleistes*. The latter clade is a robustly supported (BS 100) monophyletic group. In accordance with all individual molecular analyses, *C. tenuis* is basal in the tropical *Cleistes* clade (Fig. 1). *Cleistes libonii* and *C. metallina* are strongly supported (BS 100) as sister species. *Cleistes paranaensis* is sister to a group strongly supported as monophyletic (BS 100), having *C. bella* as basal taxon. Inside this group, well-supported clades are (*C. aphylla*+ *C. pusilla*), (*C. itatiaiae* (*C. cipoana* + *C. exilis*)), and (*C. gracilis* (*C. ramboi* + *C. uliginosa*)), all with BS 100 (Fig. 1).

Fig. 1 contains information regarding relationships of species for which sequences were obtained for one or several but not all markers, and which were not included in the analysis of the combined data. These species are (markers analyzed in parenthesis): *Cleistes caloptera* (*rbcL*), *C. castanoides* (*psaB*, *rbcL*, *rps16*, *trnL-F*), *C. divaricata* (ITS, *psaB*, *rbcL*, *trnL-F*), *C. moritzii* (*psaB*, *rps16*, *trnL-F*), *C.* aff. *ramboi* (*psaB*, *rbcL*), *C. rosea* (*psaB*, *rbcL*), *Epistephium lucidum* (*psaB*, *rbcL*), *E. parviflorum* (*psaB*), *E. sclerophyllum* (*rbcL*, *trnL-F*), *Isotria modeoloides* (*psaB*, *rbcL*), *Pogonia japonica* (ITS, *psaB*, *rbcL*), *P. minor* (ITS, *psaB*, *rbcL*), *Pogoniopsis nidus-avis* (ITS), *Vanilla bahiana* (ITS, *rbcL*, *trnL-F*), *V. palmarum* (*psaB*), and *V. pompona* (ITS, *trnL-F*). Inclusion of these species makes Fig. 1 a complete representation of phylogenetic relationships inferred in the present work.

Discussion

Phylogeny within tribe Pogonieae

Cameron and Chase (1999) and Cameron et al. (1999) regarded tribe Pogonieae as a monophyletic group, but the South-American *Pogoniopsis* was not included in their analyses. In the present study, inclusion of *Pogoniopsis* was possible only for ITS, resulting in a position at the base of the Pogonieae clade (Fig. 1). In contrast, non-molecular characters suggest that the inclusion of this genus of saprophytic plants makes the tribe paraphyletic (Pansarin 2005). A non-molecular tree shows that *Pogoniopsis* is related more closely to *Galeola* and *Cyrtosia* of Galeolinae (tribe Vanilleae; sensu Cameron 2003) than to the genera currently recognized in Pogonieae. *Pogoniopsis* contains two saprophytic species with sympodial habit, rudimentary leaves, terminal inflorescences, absence of an abscission layer between flower and ovary, fleshy and yellow indehiscent fruits, and sclerified seeds (Pansarin 2005). All these characters are shared with species from

subtribe Galeolinae (Dressler 1993), i.e. tribe Vanilleae (Cameron 2003).

All analyses assign to *Duckeella* a basal position within Pogonieae (Fig. 1), in accordance with recent morphological and molecular studies (Dressler 1993; Cameron and Chase 1999; Cameron et al. 1999; Pansarin 2005). *Duckeella*, however, has been described as lacking the defining synapomorphies of tribe Pogonieae, by having densely fasciculate and numerous fibrous roots, solid stems with basally linear leaves, lateral, ramified and multi-flowered inflorescences, yellow flowers, and a lip poorly differentiated from the petals. According to Szlachetko (1995), *Duckeella* is distinct from the remaining genera of Pogonieae (i.e. subtribe Pogoniinae), which prompted the author to ranking it at subtribe level (Duckeellinae).

The genera *Isotria*, *Pogonia* and *Cleistes* form a monophyletic unit (Fig. 1) characterized by fibrous roots, hollow stems, generally elliptic, lanceolate or ovate leaves, terminal and unbranched inflorescences with one to few white, pink or violet flowers, generally with a tubular lip and a long and parallel-orientated column. Although Cameron and Chase (1999) and Cameron et al. (1999) regard the North American–Asiatic species of Pogonieae as most derived within the subtribe, all analyses of the present work indicate that the temperate clade is sister to the tropical *Cleistes* clade (see Fig. 1).

All isolated and combined analyses show that *Cleistes* is a paraphyletic genus. In agreement with findings of Cameron and Chase (1999) and Cameron et al. (1999), the North American *C. bifaria* and *C. divaricata* form a monophyletic group with the North American *Isotria* and the North-American–Asiatic *Pogonia* in the present analyses. The South and Central American *Cleistes* form a monophyletic unit supported by both morphological (Pansarin 2005) and DNA sequence data (Fig. 1). *Cleistes* is characterized by tuberous roots, nectar production, and South and/or Central American distribution. Synapomorphies joining *Isotria verticillata* with the North American *Cleistes* and the North American–Asiatic *Pogonia* are a chromosome number of $2n = 18$, sinuous epidermal cells, hypostomatous leaves, and a North American–Western Asian distribution (Cameron and Chase 1999).

Phylogeny within the South and Central American *Cleistes* clade

The phylogenetic analyses based on the *trnL-F* and *psaB* regions yielded a tree with several polytomies inside the tropical *Cleistes* clade. On the other hand, the individual ITS, *rps16* and *rbcL* analyses and the combined molecular analysis resulted in trees with better-resolved clades. The present results are not congruent with the species "alliances" proposed by Hoehne (1940) and Pabst and Dungs (1975) in their treatments of the Brazilian orchids. Traditionally, species of *Cleistes* have been ordered artificially, the classification of the Brazilian taxa having been based mainly on differences in lip form and size (Hoehne 1940; Pabst and Dungs 1975). Evidence from recent studies by Pansarin and Barros (unpublished) agrees with the present results and shows that such characters are not consistent with species delimitation within *Cleistes*.

The present work assigns a basal position to the widely distributed (Amazon to South Brazil) marshland-inhabiting *Cleistes tenuis* (Fig. 1). In some individual analyses this species is close to *C. rosea* and *C. castanoides*. *Cleistes tenuis* contains small plants with delicate white flowers with a 3-lobed lip, whereas *C. rosea* and *C. castanoides* are large plants with large and generally pink flowers having a 1-lobed lip. These three most basal South–Central American species of *Cleistes* are also distributed at higher latitudes, with *C. rosea* reaching into Central America. The remaining species occur mainly in central-western and southeastern Brazil. The ancestor of the *Cleistes* clade probably descended from a south-migrating North American lineage: The North American–Asiatic clade is basal relative to the tropical *Cleistes* clade (Fig. 1). The center of diversity of *Cleistes* is in Central Brazil (Hoehne 1940; Pansarin 2005). According to Cameron and Chase (1999), Pogonieae originated in the South American Guiana Shield during the Late Cretaceous, and the ancestor of the present North American clade migrated to southeastern North America during the Paleocene. As no direct land connections between North and South America existed at that time (Raven and Axelrod 1974), the initial invasion of North America and later of Central and South America might be accounted for by long-distance dispersal – a reasonable assumption given their dust-like seeds (Dressler 1993).

Clade A in Fig. 1 comprises *Cleistes metallina* and *C. libonii*, both included in the same species "alliance" by Hoehne (1940) and Pabst and Dungs (1975). Main synapomorphies of these two species are large habit, conspicuous leaves and large, pink flowers. *Cleistes libonii* is distinct from *C. metallina* by the presence of dark veins on the lip and by distribution in Atlantic Forests, mainly along road margins (Pansarin 2003), whereas *C. metallina* occurs in wet fields adjacent to cerrado vegetation. Synonyms of *C. libonii* are *C. revoluta* (Barb. Rodr.) Schltr., *C. macrantha* (Barb. Rodr.) Schltr., and *C. magnifica* (Schltr.) Schltr.; *C. caloptera* (Rchb. f. & Warm.) Schltr. is synonymous with *C. metallina* (Pansarin 2005). Clade A is sister to *C. paranaensis*, which grows mainly in Central Brazil, on cerrado dry soils, having large pink flowers with long hairs on the central crest and discrete veins on the lip. *Cleistes paranaensis* is sister to the foliose *C. bella* with

vinaceous large flowers. This species also occurs in cerrado vegetation with grasslands and shrubs in Central and Southeast Brazil (Brasília, Goiás and Minas Gerais). *Cleistes bella* is sister to a clade containing the inner clades B, C and D in Fig. 1. Clade B comprises *C. aphylla* and *C. pusilla*, both small plants with scale-like leaves and 1 or 2 delicate flowers. Reduced leaves, as in *C. aphylla* and *C. pusilla*, seem to have evolved more than once within Central and South American species, since *C. paranaensis* also has reduced leaves. *Cleistes pusilla* is a rare species distributed in Central Brazil frequently misidentified as *C. aphylla* (Pansarin 2004). *Cleistes pusilla* is clearly distinct from *C. aphylla* by its larger and pink flowers, purple veins on the lip, and by a large central crest formed by white papillae and yellow apices (Pansarin 2004). The presence of white flowers in *C. aphylla*, which emerges as the most derived *Cleistes* species in all individual analyses, seems to be a reversal, since the basal *C. tenuis* also has whitish flowers. Clade C contains *C. itatiaiae* and the closely related *C. cipoana* and *C. exilis. Cleistes itatiaiae* occurs in higher-altitude fields of South and Southeast Brazil; its flowers have a large lip without dark veins and with a small apical lobe. *Cleistes cipoana* and *C. exilis*, the latter with reduced leaves, have dark veins on the lip, and occur in wet fields in the Brazilian states of Bahia, Goiás and Minas Gerais. Clade D includes *C. gracilis*, widely distributed in quartzite islands of Brazilian mountain regions. *Cleistes gracilis* is morphologically similar to *C. ionoglossa* (Pansarin 2005; Pansarin and Barros, unpublished). Clade D also includes the closely related *C. ramboi* and *C. uliginosa. Cleistes uliginosa* occurs mainly in wet fields adjacent to gallery forests and has falcate lateral lobes of the lip, a long prolongation between the lateral and apical lobes, and a lip with dark and conspicuous veins; *C. ramboi* occurs mainly in quartzite islands in Brazilian mountain regions. *Cleistes ramboi* and *C. uliginosa* are separated by the presence of hairs in the former, papillae in the latter, on the apical portion of the central crest.

Cleistes comprises terrestrial herbs growing mainly in grasslands. This condition appears to have originated once in a North American–Asiatic clade, since species of *Isotria* (Mehrhoff 1983), *Pogonia* (Thien and Marcks 1972), as well as *Cleistes divaricata* and *C. bifaria* (Gregg 1989) all grow in that type of habitat. All individual and combined analyses (Fig. 1) suggest a South American origin for Pogonieae, from where the ancestor of the North American clade migrated to the southeastern United States. *Pogonia* shows a North American–Asian disjunction (Cameron and Chase 1999). Whereas the origin of the tropical *Cleistes* seems to be well established (from a North American ancestor), the biogeography within the group is not clear. Many species share the same habitat and have overlapping distributions. Furthermore, seeds of *Cleistes* species are dust-like and wind-dispersed (Dressler 1993; Cameron and Chase 1998; Pansarin 2005).

Establishing taxonomic and evolutionary relationships among tropical *Cleistes* also is difficult. Flower traits are relatively constant among South and Central American species (Hoehne 1940; Pabst and Dungs 1975; Pansarin 2005). All taxa have flowers adapted to pollination by nectar-seeking bees (Pansarin 2003), i.e. tubular flowers with nectar guides and nectaria at the lip basis, with differences in some specific floral characters and flower size (Hoehne 1940; Pabst and Dungs 1975; Pansarin 2005). Lack of a calibration point precluded a molecular clock approach in the present study. Nonetheless, a recent radiation of tropical *Cleistes*, as suggested by Hoehne (1940), seems likely.

Tropical *Cleistes* shows marked differences in rates of morphological and molecular evolution, as also documented for other orchid groups (Ponsie et al. 2007). Thus, additional morphological, chemical and ecological data are needed to achieve a more accurate picture of the phylogeny and character evolution in the taxonomically problematical tropical *Cleistes* clade.

Taxonomic implications

All analyses of the present investigation provide evidence of close phylogenetic relationships among *Cleistes divaricata*, *C. bifaria*, *Isotria* and *Pogonia* (Fig. 1). This fact points to the necessity of taxonomic realignments in *Cleistes*. Segregation of *C. divaricata* and *C. bifaria* from *Cleistes* is phylogenetically and biogeographically meaningful. The type species of *Cleistes* is *C. grandiflora* (Aubl.) Schltr., a tropical species distributed in South and Central America. Realignment in *Cleistes* was previously suggested by Cameron and Chase (1999), with two alternative proposals: (1) establishment of a new genus for North American *Cleistes*, or (2) assembling *C. divaricata*, *C. bifaria*, *Isotria* and *Pogonia* into a single genus, *Pogonia* Juss. Those authors, however, commented that such changes could not be made until studies with additional *Cleistes* species were done. The inclusion of most South and Central American *Cleistes* in the present molecular analyses provides strong evidence that South and Central American *Cleistes* form a monophyletic tropical group distinct from its temperate congeners, as previously suggested by Cameron et al. (1999). Of the two alternatives put forward by Cameron and Chase (1999), we prefer the creation of a new genus for *C. divaricata* and *C. bifaria*, because *Isotria* with its whorled leaves and *Pogonia* with its long hairs on the lip are distinct from *Cleistes* (Pansarin 2005). This proposal does not conflict with molecular evidence (Fig. 1).

Within South and Central American *Cleistes*, *C. cipoana* consistently groups with *C. exilis* (Fig. 1).

Morphologically, these two species are similar and treated as conspecific in a taxonomic revision of *Cleistes* (Pansarin and Barros, unpublished). Similar comments apply to *C. libonii* and *C. metallina*, and to *C. ramboi* and *C. uliginosa* (Fig. 1). *Cleistes itatiaiae* is either close to or synonymous with *C. mantiqueirae*. Further morphological and molecular studies are necessary to come to a decision here. The phylogenetic proximity among South and Central species of *Cleistes* will probably play an important role in the revision of this genus (Pansarin and Barros, unpublished).

Morphological synapomorphies in Pogonieae

The present molecular analyses resulted in consistent relationships within South and Central American *Cleistes* (Fig. 1). The relationships among genera do not disagree with a non-molecular analysis (Pansarin 2005). The latter was important for the establishment of morphological synapomorphies aiming at the recognition of species groups coincident with clades in the molecular tree (Fig. 1), and for determining the position of Pogonieae among vanilloid orchids. Morphological synapomorphies for Pogonieae, *Duckeella* excluded, are: fibrous roots, hollow stems, terminal inflorescences with few flowers with white, pink or violet perianth parts (Fig. 1). Synapomorphies for temperate Pogonieae are: chromosome number $2n = 18$, epidermal cells sinuous, and North American–Eastern Asian distribution (Fig. 1). Tropical Pogonieae share the following synapomorphies (Fig. 1): root tuberous, nectar production, Central and South American distribution.

Consideration of characters from both morphology and DNA sequences were shown to be relevant also in, e.g. *Bifrenaria* (Koehler et al. 2002). Saprophytic orchids, such as some basal Epidendroideae and vanilloid orchids (Dressler 1993), including the South American *Pogoniopsis* of the present investigation, seem to have lost many of the cpDNA regions currently used in systematic studies (but see Cameron (2004) and his findings about the saprophytic *Cyrtosia*). Thus, the use of nuclear markers (e.g. ITS) and adequate morphological and anatomical studies are important to establish phylogenetic and taxonomic relationships among achlorophyllous orchids. Although the use of ITS has been challenged due to paralogy in some plant groups (Bailey et al. 2003), and morphology has been incongruent with molecular phylogeny in some orchid studies (e.g. van den Berg et al. 2000; Kores et al. 2001; Pridgeon et al. 2001), in Pogonieae these non-plastid markers seem to be valuable for phylogenetic and taxonomic studies (Cameron and Chase 1999; Pansarin 2005). Indeed, the present study shows that, at least for work at the generic level, ITS generated trees that did not conflict with other molecular markers. This is promising for phylogenetic and systematic studies of the vanilloid group, and hence for the understanding of the evolution of the huge and diverse orchid family.

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