Plant Syst Evol (2009) 283:165–177
DOI 10.1007/s00606-009-0224-2

ORIGINAL ARTICLE

Phylogenetic relationships and infrageneric classification of *Epidendrum* subgenus *Amphiglottium* (Laeliinae, Orchidaceae)

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Received: 18 April 2009 / Accepted: 14 September 2009 / Published online: 25 September 2009

Abstract *Epidendrum* L. is the largest genus of Orchidaceae in the Neotropical region; it has an impressive morphological diversification, which imposes difficulties in delimitation of both infrageneric and interspecific boundaries. In this study, we review infrageneric boundaries within the subgenus *Amphiglottium* and try to contribute to the understanding of morphological diversification and taxa delimitation within this group. We tested the monophyly of the subgenus *Amphiglottium* sect. *Amphiglottium*, expanding previous phylogenetic investigations and reevaluated previous infrageneric classifications proposed. Sequence data from the *trn*L-*trn*F region were analyzed with both parsimony and maximum likelihood criteria. AFLP markers were also obtained and analyzed with phylogenetic and principal coordinate analyses. Additionally, we obtained chromosome numbers for representative species within the group. The results strengthen the monophyly of the subgenus *Amphiglottium* but do not support the current classification system proposed by previous authors. Only section *Tuberculata* comprises a well-supported monophyletic group, with sections *Carinata* and *Integra* not supported. Instead of morphology, biogeographical and ecological patterns are reflected in the phylogenetic signal in this group. This study also confirms the large variability of chromosome numbers for the subgenus *Amphiglottium* (numbers ranging from $2n = 24$ to $2n = 240$), suggesting that polyploidy and hybridization are probably important mechanisms of speciation within the group.

Keywords Orchidaceae · *Epidendrum* · Subgenus *Amphiglottium* · *trn*L-*trn*F intergenic spacer · AFLP marker

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Introduction

Epidendrum L. is the largest genus of Orchidaceae in the Neotropical region, with about 1,500 species distributed from the southeastern United States to northern Argentina (Hágsater and Soto Arenas 2005). The genus shows an impressive morphological diversification, with an extensive number of plesiomorphies that make it difficult to limit generic and infrageneric boundaries. For some groups, such as subgenus *Amphiglottium*, even interspecific boundaries are difficult to ascertain. Studies on *Epidendrum* have been focussing on the description of new species whereas complementary taxonomic and evolutionary studies are much more restricted (see review by Hágsater and Soto Arenas 2005). Phylogenetic studies indicate that *Epidendrum*, including *Oerstedella* and *Neowilliamsia*, is monophyletic (van den Berg et al. 2000; Hágsater and Soto Arenas 2005).

According to van den Berg et al. (2000) and Hágsater and Soto Arenas (2005), several informal species groups within *Epidendrum*, traditionally recognized by morphological characters (Hágsater 1984), were also confirmed as well-supported monophyletic groups. One of such clades is

the subgenus *Amphiglottium* sect. *Amphiglottium* (Salisb.) Brieger, which is characterized by reed-like stems, elongate peduncles of the inflorescences with close-fitting sheaths, racemes usually congested or subcorymbose with flowers densely clustered at the end of the peduncle. Morphological cohesion in subgenus *Amphiglottium* sect. *Amphiglottium* concurs with ecological traits, such as the ability to grow in very disturbed sites, such as roadside banks, lava flows, sand dunes and lake beds (Hágsater 1984). The rapid germination and growth rates observed in species of this group also suggest they are highly competitive (Hágsater and Soto Arenas 2005).

Hybridization events in the group may be a common phenomenon and add additional challenges to infrageneric classification, as hybridization can occur among species from different groups (Dunsterville 1979; Hágsater 1984; Dressler 1989). Hybridization can be the origin of the extensive morphological variation and of the differences in chromosome numbers observed in some species complexes co-occurring in the same localities. High levels of reproductive compatibility in the subtribe Laeliinae, to which *Epidendrum* belongs (Lenz and Wimber 1959; Pansarin and Amaral 2008), and the lack of specific pollinators among *Epidendrum* species (Van der Pijl and Dodson 1966; Pansarin and Amaral 2008) indicate weak or absent pre-pollination barriers, making hybridization in natural populations a feasible event. Human activities are reported as the main origin of some hybrid swarms, as many species pairs come in contact after road constructions, occupying roadside cuts and deforested slopes (Dunsterville 1979; Hágsater and Soto Arenas 2005; Pansarin and Amaral 2008). Despite the wide range of reports of hybridization in *Epidendrum*, none has been examined with molecular markers.

Species of the subgenus *Amphiglottium* were first recognized by Lindley (1852–1859) as belonging to *Epidendrum* subgenus *Amphiglottium*. The 34 species at the time were also divided by him into three sections (*Schistochila integra*, *Schistochila carinata*, and *Schistochila tuberculata*), according to features of the lip margin and shape of callus (Table 1). Cogniaux (1898–1902), Pabst and Dungs (1975) and Brieger (1976–1977) proposed alternative classification systems for the subgenus *Amphiglottium* that repeated, to a great extent, Lindley's classification. Cogniaux (1898–1902) put all the species from Lindley's subgenus *Amphiglottium* in the subsection Amphiglottidae, but recognized the same three sections from Lindley in his identification key for species as informal subdivisions of subsect. *Amphiglottidae*. Pabst and Dungs (1975) considered Cogniaux's subsection *Amphyglottidae* and three alliances (*E. polyanthum*, *E. schomburgkii*, and *E. denticulatum*) defined by the same diagnostic characters of Lindley's sections. Brieger (1976–1977) also based his classification on Lindley's work but named the subsections within subgenus *Amphyglottium* as subsect. *Integra*, subsect. *Carinata,* and subsect. *Tuberculata* (Table 1). Species from subsect. *Integra* have greenish flowers with brown spots, while species belonging to the remaining two subsections, *Carinata* and *Tuberculata*, have flowers ranging from white, yellow, red, orange, and pink (Brieger 1976–1977; Table 1).

Within the *Tuberculata* subsection, the *Epidendrum secundum* complex is one of the less taxonomically understood groups (Brieger 1976–1977; Dunsterville 1979). They are extremely polymorphic plants (Pinheiro and Barros 2008) and thoroughly distributed in Central and South America. The complexity of the group is reflected in the great number of names associated with it, sometimes considered as synonyms of *E. secundum*, sometimes as autonomous species (*E. elongatum* Jacq., *E. crassifolium* Lindl., *E. ellipticum* Grah., *E. ansiferum* Rchb. f., *E. versicolor* Hoehne & Schltr., among others). Each author has a point of view on how to deal with the variations presented by the species that compose the *E. secundum* complex: some considering each morpho or flower coloration as belonging to a different species (Pabst and Dungs 1975; Brieger 1976–1977), and others considering that the variations are continuous and are part of the same polymorphic species (Dressler 1989; Dunsterville and Garay 1961). Uncertainties concerning the species delimitation of the group still remain as the available studies are based on regional assessments and on morphological characters only.

Flower morphological features are largely used by taxonomists when proposing new classifications for Orchidaceae, especially at the genus and species level, as flowers are the most conspicuous part of the plant and the richest in characters. Not surprisingly, the extensive flower variation found in *Epidendrum*, and the homogeneity in vegetative morphology encouraged the proposition of many new sections and subsections that were based exclusively on flower traits (Lindley 1852–1859; Brieger 1976–1977). Classifications based solely on morphological characters are prone to include a large proportion of nonphylogenetic information due to convergence (Funk and Omland 2003). In orchids there is strong evidence that flower morphology is correlated with both ecological factors and particular life-history traits in addition to phylogeny (Singer and Koehler 2004). Available phylogenetic studies for orchid genera presenting high diversification of flower morphology, such as *Orchis* (Aceto et al. 1999), *Pleurothallis* (Borba et al. 2002), and *Maxillaria* s.l. (Whitten et al. 2007), revealed artificial classifications based mainly on the convergence of flower morphological characters. The question of how informative flower characters in *Epidendrum* are remains unanswered, even though available

Table 1 Summary of the former infrageneric classifications proposed for the subgenus *Amphiglottium*

Author	Main taxonomic group	Diagnostic characters	First subdivision	Diagnostic characters	Second subdivision	Diagnostic characters	Number of species
Lindley (1852–1859)	Subgenus *Amphyglottium*	Long flower racemes with close-fitting sheaths	Section *Schistochila*	Long inflorescence, flowers concentrated on the top	Subsection *integra*	Lip margins not crenate	14
					Subsection *carinata*	Lip margins crenate, central lip callus in a ridged line shape, projected longitudinally on the disc	7
					Subsection *tuberculata*	Lip margins crenate, thick, lip callus unlobed	13
Cogniaux (1898–1902)	Subsection *Amphiglottidae*	Simple stem, coriaceous leaves, long inflorescences	No further subdivisions, but diagnostic characteristics in the identification key were based on Lindley's classification	Not presented	–	–	21 (only in Brazil)
Pabst and Dungs (1975)	Amphyglottidae group	Long inflorescences, as the size of the stem	*Epidendrum polyanthum* alliance	Lip margins not crenate	–	–	5 (only in Brazil)
			Epidendrum schomburgkii alliance	Lip margins crenate, central lip callus in a ridged line shape, projected longitudinally on the disc	–	–	7 (Only in Brazil)
			Epidendrum denticulatum alliance	Lip margins crenate, thick, lip callus unlobed	–	–	11 (Only in Brazil)
Brieger (1976–1977)	Subgenus *Amphyglottium*	Inflorescence with peduncle much longer than the flowered portion and covered by sheaths	Section *Amphyglottium*	Long inflorescence, flowers concentrated on the top	Subsection *Integra*	Flowers greenish with brown spots; callus with a thick central line and two basal rounded protuberances	6
					Subsection *Carinata*	Flowers with lip margins strongly dentate to fringed; callus with a central, sharp-pointed, generally wavy lamella	11
					Subsection *Tuberculat*a	Callus with various forms, but essentially a central crest with two basal protuberances, sometimes without these later ones	4

classification systems within the genus are mostly based on them, as observed for the subgenus *Amphiglottium*.

Molecular phylogenies have been widely used to test formal classification systems based only on morphological characters as well as to improve them (Chase 1999). Nonetheless, available phylogenetic studies for *Epidendrum* have considered very few species of the subgenus *Amphiglottium*. In the first phylogenetic studies for the subtribe Laeliinae, van den Berg et al. (2000) sampled three species (*E. secundum, E. cinnabarinum*, and *E. ibaguense*), while Hágsater and Soto Arenas (2005) presented a tree in which the subgenus *Amphiglottium* includes *E. smaragdinum*, *E. ibaguese*, *E. radicans,* and *E. thermophilous* with 100% of bootstrap support (ITS + matK). Unpublished data from Hágsater and Soto Arenas, based on the ITS-region, show ([(*E. ibaguense* + *E. thermophilous*) + (*E. radicans* + *E. flexuosum*)] + (*E. orchidiflorum* + *E. smaragdinum*)), with all internal clades with bootstrap values higher than 93% and *E. blepharistes* sister to the ingroup with 97% bootstrap (Hágsater and Soto Arenas, pers. comm.). In total eight species were analyzed in a phylogenetic context out of the 21 estimated for the group by Brieger (1976–1977).

Studies on additional characters that might be useful for understanding patterns of diversification in this group are also scanty. The few chromosome counts available for species of the subgenus *Amphiglottium* indicate high intra- and interspecific variability. Interspecific studies report diploid numbers ranging from $2n = 24$ (Blumenschein 1960) to $2n = 240$ (Conceição et al. 2006). Additionally, diploid numbers of $2n = 38$, 40, 48, 60, 62, 64, and 70 have been reported for *E. radicans* (Tanaka and Kamemoto 1984) and $2n = 40$, 60, and 80 for *E. xanthinum* (Tanaka and Kamemoto 1984; Vij and Shekhar 1985), with the basic number reported for the genus *Epidendrum* as being $2n = 40$ (Hágsater and Soto Arenas 2005).

Sequencing of DNA regions has been successfully applied widely in the phylogenetic reconstructions at species and generic levels in many plant groups, including orchids (e.g., Aceto et al. 1999; van den Berg et al. 2000). Nevertheless, the lack of adequate variability among recently radiated taxa represents a major limitation of DNA sequence data for resolving phylogenetic relationships (e.g., Després et al. 2003). Alternatively, dominant markers comprise an alternative source of information for phylogenetic and systematic studies of closely related species when theoretical concerns and limits of their application are addressed (Després et al. 2003; Bussell et al. 2005; Archibald et al. 2006).

Our goal in this study was to review infrageneric boundaries within the subgenus *Amphiglottium* sect. *Amphiglottium*, as circumscribed by Brieger (1976–1977) and contribute to understanding the morphological diversification and taxa delimitation within this group. In order to achieve this, we aim to (1) test the monophyly of the subgenus *Amphiglottium*, expanding previous phylogenetic investigations and (2) reevaluate the previous infrageneric classifications proposed, using molecular data. We also obtained chromosome numbers for representative species within this group in order to explore the patterns of variation in a phylogenetic framework.

Materials and methods

Sampling

Species identification followed Hágsater and Salazar (1990), Carnevali and Ramírez-Morillo (2003), Vasquez and Ibisch (2004), and Pinheiro and Barros (2006; 2007). Voucher specimens of the examined plants were deposited at the Herbarium of the Instituto de Botânica SP (Table 2).

In order to test the monophyly of the subgenus *Amphiglottium*, we sequenced the *trn*L-*trn*F chloroplast intergenic spacer for 25 individuals and 13 species (Table 1). For the evaluation of previous classifications for this group, we expanded the taxon sampling for the AFLP analyses to 49 individuals (Table 1). Outgroup species used were *Epidendrum campestre*, *E. cristatum*, *E. nocturnum*, *E. saxatile*, and *E. smaragdinum*, according to the most recent phylogenetic study available for *Epidendrum* (Hágsater and Soto Arenas 2005). To avoid problems with long branch attraction in the AFLP analyses (Smith 1994), only *E. nocturnum* and *E. smaragdinum* were used as outgroups since current available phylogenetic studies place both species as closely related to the ingroup (Hágsater and Soto Arenas 2005).

DNA extraction and sequence data

Total genomic DNA was extracted from fresh tissue following the protocol of Doyle and Doyle (1990). The *trn*L-*trn*F region was amplified using the primers C and F described in Taberlet et al. (1991). PCR amplifications were performed in an Eppendorf thermocycler in 50-μL reactions containing the following: 10 ng template, 1× PCR buffer (Invitrogen, São Paulo, Brazil), 2.5 mM $MgCl_2$, 100 μM dNTPs, 0.5 μM of each primer, and 0.5 U Taq polymerase Platinum (Invitrogen). The cycle profile was 95°C for 4 min; then 30 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 2 min; followed by a final extension of 10 min at 72°C. Amplification products were then purified (Qiaquick PCR purification Kit, QIAGEN Biotecnologia Brasil). The sequencing reactions were performed in a total volume of 10 μL containing 30–50 ng DNA, 5 μM of each primer, 2 μL of the ABI PRISM

Table 2 Specimens with voucher information and GenBank accession numbers considered in molecular studies

Species (code)	Collection location	Voucher	GenBank Accession
E. calanthum Rchb. f. and Warsc. (BR)	Brazil, Serra Pacaraima	*F. Pinheiro 525* (A)	
E. calanthum (EQ)	Ecuador	*F. Pinheiro 524* (A, S)	FJ869393
E. campestre Lindl.	Brazil, Santana do Riacho	*F. Pinheiro 572* (A, S)	FJ869394
E. cinnabarinum Salzm. (Sal)	Brazil, Salvador	*F. Pinheiro 540* (A)	
E. cinnabarinum (Fel)	Brazil, Camocim de São Felix	*F. Pinheiro 539* (A, S)	FJ869395
E. cochlidium Lindl. (PE)	Peru	*F. Pinheiro 557* (A, S)	FJ869422
E. cochlidium (EQ)	Ecuador, Oxapampa	*F. Pinheiro 556* (A, S)	FJ869423
E. cristatum Ruiz and Pav.	Brazil	*F. Pinheiro 573* (A, S)	FJ869396
E. denticulatum Barb. Rodr. (Gon)	Brazil, São Gonçalo do Rio Abaixo	*F. Pinheiro 542* (A, S)	FJ869397
E. denticulatum (Del)	Brazil, Delfinópolis	*F. Pinheiro 541* (A)	
E. flexuosum G.Mey	Brazil, Mamirauá	*F. Pinheiro 575* (S)	FJ869421
E. fulgens Brongn. (Par)	Brazil, Parati	*F. Pinheiro 523* (A, S)	FJ869398
E. fulgens (Can)	Brazil, Cananéia	*F. Pinheiro 543* (A)	
E. ibaguense H.B.K (Ror2)	Brazil, Serra Pacaraima	*F. Pinheiro 526* (A)	
E. ibaguense (Ror1)	Brazil, Serra Pacaraima	*F. Pinheiro 555* (A, S)	FJ869399
E. incisum Vell. (EQ)	Ecuador	*F. Pinheiro 532* (A, S)	FJ869400
E. incisum (BR)	Brazil, Congonhas do Norte	*F. Pinheiro 533* (A)	
E. myrmecophorum Barb. Rodr. (Sal)	Brazil, Salvador	*F. Pinheiro 527* (A, S)	FJ869401
E. myrmecophorum (Cha)	Brazil, Chapada Diamantina	*F. Pinheiro 531* (A)	
E. nocturnum Jacq.	Brazil, Diamantina	*F. Pinheiro 574* (A, S)	FJ869420
E. puniceoluteum F.Pinheiro and F.Barros (Can)	Brazil, Cananéia	*F. Pinheiro 529* (A)	
E. puniceoluteum (Com)	Brazil, Ilha Comprida	*F. Pinheiro 528* (A, S)	FJ869402
E. purpureum Barb. Rodr.	Brazil, Araruama	*F. Pinheiro 530* (A, S)	FJ869403
E. radicans Pav. ex Lindl. (ME)	Mexico, Oaxaca	*F. Pinheiro 549* (A, S)	FJ869404
E. radicans (EQ)	Ecuador	*F. Pinheiro 554* (A)	
E. saxatile Lindl.	Brazil, Santana do Riacho	*F. Pinheiro 576* (S)	FJ869405
E. secundum Jacq. lilac flower (lfBO1)	Bolivia	*F. Pinheiro 550* (A, S)	FJ869406
E. secundum lilac flower (lfBO2)	Bolivia	*F. Pinheiro 551* (A)	
E. secundum lilac flower (lfBR1)	Brazil, Santa Bárbara	*F. Pinheiro 552* (A)	
E. secundum lilac flower (lfBR2)	Brazil, Diamantina	*F. Pinheiro 553* (A, S)	FJ869407
E. secundum lilac flower (lfBR3)	Brazil, Santo Antonio do Itambé	*F. Pinheiro 558* (A)	
E. secundum lilac flower (lfBR4)	Brazil, Nova Friburgo	*F. Pinheiro 559* (A)	
E. secundum lilac flower (lfBR5)	Brazil, Pindamonhangaba	*F. Pinheiro 537* (A, S)	FJ869408
E. secundum lilac flower (lfBR6)	Brazil, Santana do Riacho	*F. Pinheiro 538* (A)	
E. secundum lilac flower (lfBR7)	Brazil, Serra do Rio do Rastro	*F. Pinheiro 560* (A, S)	FJ869409
E. secundum lilac flower (lfVE1)	Venezuela, Monte Roraima	*F. Pinheiro 561* (A, S)	FJ869419
E. secundum lilac flower (lfVE2)	Venezuela	*F. Pinheiro 534* (A, S)	FJ869410
E. secundum orange flower (ofBR1)	Brazil, Santo Antonio do Itambe	*F. Pinheiro 535* (A)	
E. secundum orange flower (ofBR2)	Brazil, Santo Antonio do Itambe	*F. Pinheiro 562* (A)	
E. secundum orange flower (ofBR3)	Brazil, Nova Friburgo	*F. Pinheiro 563* (A)	
E. secundum red flower (rfBR)	Brazil, Nova Friburgo	*F. Pinheiro 536* (A, S)	FJ869411
E. secundum red flower (rfEQ)	Ecuador	*F. Pinheiro 547* (A, S)	FJ869418
E. secundum white flower (wfBR1)	Brazil, Nova Friburgo	*F. Pinheiro 548* (A)	
E. secundum white flower (wfBR2)	Brazil, Paranapiacaba	*F. Pinheiro 564* (A, S)	FJ869412
E. secundum white flower (wfVE)	Venezuela	*F. Pinheiro 544* (A, S)	FJ869413
E. smaragdinum Lindl.	Brazil, Manaus	*F. Pinheiro 575* (A, S)	
E. xanthinum Lindl. (BR1)	Brazil, Santa Bárbara	*F. Pinheiro 545* (A, S)	FJ869414

Table 2 continued

Species (code)	Collection location	Voucher	GenBank Accession
E. xanthinum (BR2)	Brazil, Santa Bárbara	*F. Pinheiro 565* (A)	
E. xanthinum (BR3)	Brazil, Santa Bárbara	*F. Pinheiro 567* (A)	
E. xanthinum (BR4)	Brazil, Santo Antonio do Itambe	*F. Pinheiro 569* (A)	
E. xanthinum (BR5)	Brazil, Santo Antonio do Itambe	*F. Pinheiro 568* (A)	
E. xanthinum (BR6)	Brazil, Nova Friburgo	*F. Pinheiro 570* (A, S)	FJ869415
E. xanthinum (BR7)	Brazil, Nova Friburgo	*F. Pinheiro 571* (A)	
E. xanthinum (BR8)	Brazil, Nova Friburgo	*F. Pinheiro 566* (A)	
E. xanthinum (EQ)	Ecuador	*F. Pinheiro 546* (A, S)	FJ869416

S Sequence data, *A* AFLP data

BigDye Terminator v3.1 cycle sequencing ready reaction kit (Applied Biosystems, São Paulo, Brazil), and 1 μL of 5× Sequencing Buffer (Applied Biosystems). The thermocycling parameters were as follows: 1 cycle of 4 min at 94°C; 40 cycles at 94°C for 40 s, 52°C for 40 s, and 72°C for 1 min. PCR products were resolved on a 3700 DNA Sequence Analyser (Applied Biosystems). Sequences were edited manually using SeqMan 5.01 software (Lasergene 7.0, DNASTAR). Multiple sequence alignments were generated with MegAlign software (Lasergene 7.0, DNASTAR) using the ClustalW option following the method of Thompson et al. (1994). GenBank accession numbers are listed in Table 2.

AFLP data

AFLP markers were obtained according to the AFLP Plant Mapping Protocol (Applied Biosystems 2000). The digestion of DNA was employed with the restriction enzymes *Eco* RI and *Mse* I (Invitrogen). The resulting fragments were attached to adaptors and submitted to pre-selective and selective amplification reactions using the AFLP Plant Mapping Kit (Applied Biosystems). A total of five *Eco*/*Mse* primer combinations were used (Table 3). The samples were resolved on automatic sequencer ABI Prism 310 (Applied Biosystems). Gel analysis was carried out with Genescan 3.1 and Genotyper 2.5 (Applied Biosystems). Only amplified fragments with sizes ranging from 50 to 500 base pairs (bp) were scored as bands outside this size range cannot be accurately sized. The bands were scored as present or absent.

Chromosome counts

Root tips from 19 individuals, corresponding to 12 species (Table 4), were collected and submitted to previous treatment with 8-hydroxyquinoline 2 mM at 4°C for 24 h, and fixed for 24 h in 3:1 ethanol:glacial acetic acid at room temperature. After fixation, root tips were transferred to 70% ethanol and stored in a freezer at −18°C. Squash preparations were made in a 2% Giemsa solution (Guerra 1983) on a microscope slide. Slides were examined and documented with an optic microscope and digital camera Evolution MP 5.0 Mp.

Table 3 Total number of scored fragments and number of polymorphic fragments generated by each combination of AFLP primer pair

Primer combinations	Scored fragments	Polymorphic fragments (%)
*Eco*RI + AC − *Mse*I + CTA	55	43 (78.1)
*Eco*RI + AG − *Mse*I + CTT	53	41 (77.3)
*Eco*RI + ACT − *Mse*I + CTC	67	40 (59.7)
*Eco*RI + AAG − *Mse*I + CTA	81	25 (30.8)
*Eco*RI + ACC − *Mse*I + CAC	50	27 (54.0)
Total	306	176 (57.5)

Data analysis

Phylogenetic analyses of the *trn*L-*trn*F region were initially conducted with a heuristic search under the maximum parsimony (MP) criterion of Fitch (unordered characters, equal weights to all changes; Fitch 1971), excluding uninformative characters, and with ACCTRAN optimization. The search strategy used was 10,000 addition sequence replicates by stepwise addition holding 10 trees per replicate, TBR branch swapping on best trees, MULTREES on, saving no more than 10 trees per replicate. To assess support for internal clades, we performed 1,000 bootstrap pseudo-replicates (Felsenstein 1985) of 10 additional sequence replicates by stepwise addition holding one tree per replicate. The categories of bootstrap support considered were unsupported (<50%), weak (50–74%), moderate (75–84%), strong (85–100%). Analyses were

Table 4 Chromosome numbers for species belonging to the subgenus *Amphiglottium*

Species	Chromosome numbers (2*n*)	Voucher or reference
E. calanthum Rchb.f. & Warsc.	30	*F. Pinheiro 524*
E. cinnabarinum Salzm.	240	*Conceição* et al. (2006)
E. cochlidium Lindl.	28	*F. Pinheiro 556*
E. cochlidium Lindl.	28	*F. Pinheiro 557*
E. denticulatum Barb. Rodr.	40	*F. Pinheiro 541*
E. denticulatum Barb. Rodr.	40	Blumenschein (1960)
E. flexuosum G.Mey	28	*F. Pinheiro 575*
E. fulgens Brongn.	24	*F. Pinheiro 543*
E. fulgens Brongn.	24	Blumenschein (1960)
E. ibaguense H.B.K.	70	*F. Pinheiro 555*
E. myrmecophorum Barb. Rodr.	120	*F. Pinheiro 527*
E. puniceoluteum F.Pinheiro and F.Barros	52	*F. Pinheiro 528*
E. purpureum Barb. Rodr.	120	*F. Pinheiro 530*
E. radicans Pav. ex Lindl.	60	*F. Pinheiro 554*
E. radicans Pav. ex Lindl.	40	Tanaka and Kamemoto (1984)
E. radicans Pav. ex Lindl.	57	Tanaka and Kamemoto (1984)
E. radicans Pav. ex Lindl.	62	Tanaka and Kamemoto (1984)
E. radicans Pav. ex Lindl.	64	Tanaka and Kamemoto (1984)
E. secundum Jacq.	28	*F. Pinheiro 550*
E. secundum Jacq.	68	Blumenschein (1960)
E. secundum Jacq.	52	*F. Pinheiro 538*
E. secundum Jacq.	48	*F. Pinheiro 562*
E. secundum Jacq.	40	*F. Pinheiro 560*
E. secundum Jacq.	80	*F. Pinheiro 534*
E. xanthinum Lindl.	40	Tanaka and Kamemoto (1984)
E. xanthinum Lindl.	60	Tanaka and Kamemoto (1984)
E. xanthinum Lindl.	30	*F. Pinheiro 568*
E. xanthinum Lindl.	28	*F. Pinheiro 570*
E. xanthinum Lindl.	60	*F. Pinheiro 546*

performed in PAUP 4b10 (Swofford 2003), and trees reconstructed with the aid of Treeview (Page 1996).

For maximum likelihood analyses (ML), alternative nested models of DNA sequence data evolution were first evaluated with likelihood ratio test as implemented in MODELTEST 3.7 (Posada and Crandall 1998; $\alpha = 0.01$). The best-fit model of DNA sequence evolution with its estimated parameters was then input into detailed maximum likelihood tree searches performed in PAUP*. Starting trees were obtained using 1,000 addition sequence replicates by stepwise addition holding 10 trees per replicate, with further SPR branch swapping. Starting branch lengths were obtained using the Rogers-Swofford approximation method with branch-length optimization of Newton–Raphson. Confidence of the ML tree obtained was assessed by bootstrap analyses based on 1,000 pseudo-replicates using the fast reduced search option in PAUP*.

The binary matrix listing presence/absence of each band for all samples was created from the AFLP data and analyzed by the neighbor-joining (NJ) distance algorithm (Sneath and Sokal 1973), the maximum parsimony (MP) criterion (Fitch 1971), and the principal coordinate ordination method (PCoA, Gower 1966). The NJ analysis was carried out in PAUP4b10* (Swofford 2003) using the Nei and Li (Nei and Li 1979) distance coefficient, which measures the probability that a band being amplified in one sample is also amplified in another sample (Robinson and Harris 1999). This coefficient is also an estimate of the proportion of the number of bands shared by two samples because they were inherited from a common ancestor (Harris 1999). The search strategy for the MP and bootstrap analysis, also performed in PAUPb10*, followed the same search strategy employed for the analysis of sequence data, but using also Dollo parsimony (implemented as Dollo.Up) (Farris 1977). The Dollo parsimony method is based on the assumption that a complex character that has been lost during evolution of a particular lineage cannot be regained. The use of Dollo

parsimony has been suggested as the most suited for phylogenetic analyses of dominant markers as it considers that each apomorphic character state must be uniquely derived and that all homoplasy must be accounted for by reversals to more plesiomorphic states (Swofford and Olsen 1990; Backeljau et al. 1995). PCoA analyses were performed in Fitopac (Shepherd 2006), using Jaccard's coefficient, which excludes similarity due to shared absences.

Results

*trn*L-*trn*F sequence analyses

The MP and ML statistics for *trn*L-*trn*F phylogenetic analyses are given in Tables 5 and 6, respectively. Parsimony analyses were conducted including and excluding *E. flexuosum* due to its extremely long branch, but results indicate that the inclusion of this species did not affect the topology of the most parsimonious trees. In fact the topologies of MP and ML trees recovered in this study were extremely similar, except for the position of *E. flexuosum* (sister to *E. radicans* in the MP tree and sister to the Atlantic clade + subsection *Tuberculata* clade in the ML tree). There are also small differences regarding the topology of subsection *Tuberculata* clade between MP and ML *trnL-F* best trees, but since differences between topologies of these two optimality criteria are small, only the ML tree is shown (Fig. 1b).

The subgenus *Amphiglottium* is strongly to moderately supported as a monophyletic group (87% BS/MP; 80% BS/ML; Fig. 1b). Subsection *Carinata* is not monophyletic according to results of both MP and ML analyses (Fig. 1b). Instead, two strongly supported clades representing distinct biogeographical regions were recovered: clade III (*E. calanthum*, *E. ibaguense*, *E. incisum*; 98% BS/MP and ML) from the Andean region and clade IV (*E. cinnabarinum*, *E. fulgens*, *E. denticulatum*, *E. puniceoluteum*; 95% BS/MP; 93% BS/ML) from the Brazilian Atlantic coast. On the contrary, the monophyly of subsection *Tuberculata* was supported by the phylogenetic results based on sequence data (96% BS/MP; 94% BS/ML; clade *Tuberculata*), but with low resolution for phylogenetic relationships within this clade (Fig. 1b). The results also strongly support the subsection *Integra* sensu Brieger (1976–1977), represented by *E. myrmecophorum* and *E. purpureum*, as sister to the Atlantic, Andean, and *Tuberculata* clades (Fig. 1b). *Epidendrum radicans*, represented by a single individual and also classified in subsection *Carinata* by previous authors, was recovered as the sister species of the Atlantic, Andean, *Integra*, and *Tuberculata* clades with moderate support (Fig. 1b).

AFLP analyses

A total of 306 scorable fragments were obtained, of which 176 were variable (57.5%). As the topology of the trees obtained by Dollo MP, Fitch MP, and neighbor-joining were very similar, only results obtained by Dollo MP are show in Fig. 1a. Both Fitch and Dollo MP analyses strongly support the subgenus *Amphiglottium* as monophyletic (Fig. 1a) as well as clade *Tuberculata* (89% BS/Fitch; 100% BS/Dollo). As recovered by the sequence data, phylogenetic analyses based on AFLP data do not support subsection *Carinata* as monophyletic but strongly support both Andean and Atlantic clades (Fig. 1a). Subsection *Integra* is only weakly supported by the Dollo MP

Table 5 Tree statistics for phylogenetic analyses based on maximum parsimony criterion for *trn*L-*trn*F sequence and AFLP datasets

	MP criteria	Total no. of characters	No. of potentially parsimony-informative characters	Ingroup	Outgroup	MPT	L	CI	RI
trnL-F	Fitch	1,021	35	26	5	127	62	0.871	0.949
trnL-F (ex *E. flexuosum*)	Fitch	1,021	32	25	5	18	52	0.904	0.967
AFLP	Dollo up	193	142	49	2	73	422	0.396	0.949

Table 6 Statistics from *trn*L-*trn*F phylogenetic analyses performed under the maximum likelihood criterion

No. of ingroup taxa	Selected model	Nucleotide frequencies	Shape parameter (α value) of gamma-distributed rate variation across sites	Pinvar	-lnL value
46	F81 + G + I	A = 0.32, C = 0.15, G = 0.14, T = 0.39	0.90	0.61	10,986.45

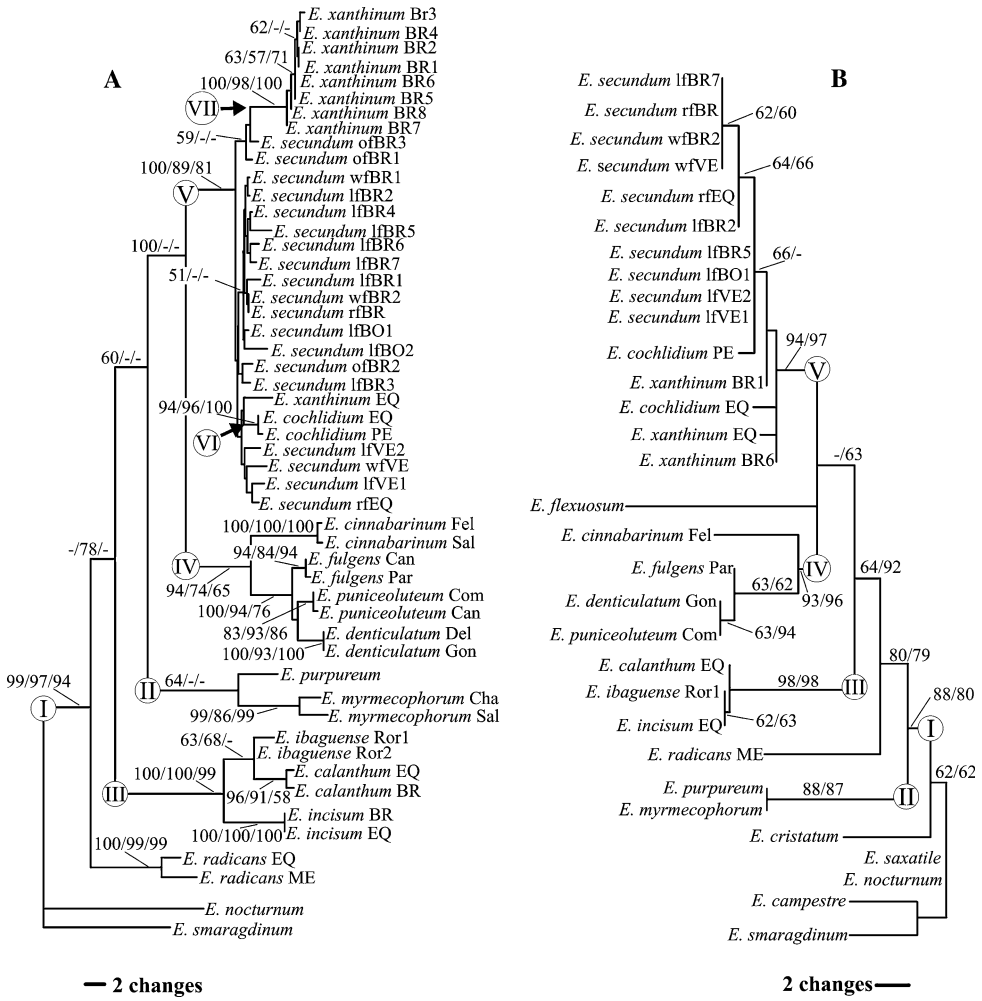

Fig. 1 One of the most parsimonious trees based on the Dollo parsimony criterion for AFLP data (**a**) and the best tree based on maximum likelihood criterion for *trn*L-*trn*F (**b**). Dollo parsimony/Fitch parsimony/neighbor-joining bootstrap support values above 50% are indicated on AFLP tree branches. Maximum likelihood/maximum parsimony bootstrap support values above 50% are indicated *above trn*L-*trn*F tree branches. Clades recognized in this study are indicated as follows: (I) *Amphyglottis* group, (II) subsection *Integra*, (III) Andean clade, (IV) Atlantic clade, (V) subsection *Tuberculata*, (VI) *E. cochlidium*, (VII) *E. xanthinum*

(64% BS) and not supported by Fitch MP. Results of the Fitch MP analysis suggested that *E. purpureum* is the sister of the Atlantic clade, with *E. myrmecophorum* as the sister to the Andean clade, but there is no BS for either result (data not shown). Deeper phylogenetic relationships supported by both AFLP and MP analyses indicate that the Atlantic clade is sister to the *Tuberculata* clade [53% BS/Fitch (incl. *E. purpureum*); 100% BS/Dollo (excl. *E. purpureum*)]. Contrary to the sequence data results, the Fitch/MP analysis recovers *E. radicans* as the sister species of other ingroup species (78% BS; not supported by Dollo MP).

Phylogenetic relationships within the *Tuberculata* clade are less resolved. The two specimens of *E. cochlidium* sampled are recovered as strongly monophyletic (Fig. 1a). Results also suggest that the Brazilian *E. xanthinum* specimens form a monophyletic group within clade *Tuberculata*, but are not phylogenetically closely related to the single specimen of *E. xanthinum* sampled from Ecuador (Figs. 1, 2). The taxonomically complex *E. secundum* is not supported as monophyletic by any data sets. Results from AFLP Fitch MP do recover *E. secundum* as monophyletic but with no bootstrap support.

Principal coordinate analysis of the subsection *Tuberculata*

In agreement with the previously presented results, based on phylogenetic analyses, the Brazilian specimens of *E. xanthinum* are more similar to each other than specimens of *E. cochlidium* and *E. secundum* in the first axis (36.58% variation) of the PCoA scatter plot (Fig. 2a). Also, in Fig. 2a, the second axis (10.44% variation) clearly distinguishes samples of *E. cochlidium* from individuals of *E. secundum* from Brazil, which comprise specimens with variable flower coloration, from a third group represented by *E. secundum* from the Andes and Guiana plus the single specimen of *E. xanthinum* sampled from Ecuador. The third axis accounted for 5.99% variation and does not clearly distinguish between specimens of *E. secundum*

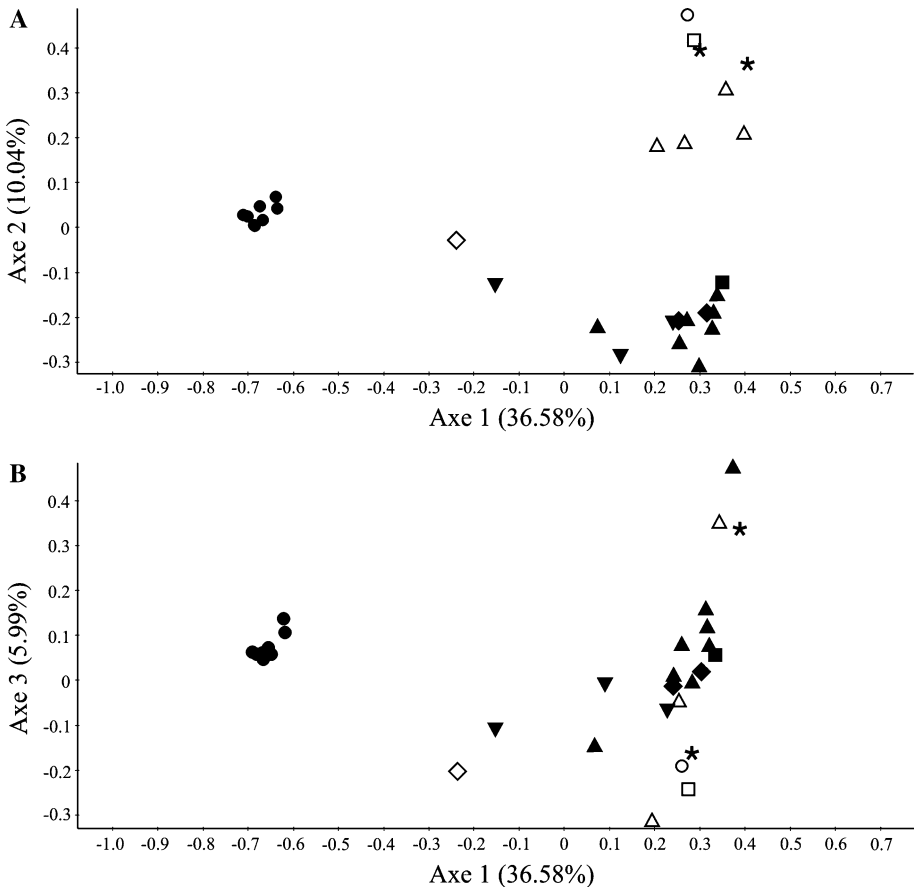

Fig. 2 Principal coordinate analysis (PCO) of AFLP data for *E. cochlidium*, *E. secundum*, and *E. xanthinum*, based on axes 1 and 2 (**a**) and 1 and 3 (**b**). *Filled symbols* Brazilian origin, *open symbols* Andean and Guianan origin, *asterisks* *E. cochlidium*, *circles* *E. xanthinum*, *triangles* *E. secundum* lilac flower, *inverted triangles E. secundum* orange flower, *squares* *E. secundum* red flower, *diamonds E. secundum* white flower

from the Andean region and Brazil or specimens of *E. cochlidium* and *E. xanthinum* from Ecuador (Fig. 2b).

Chromosome counts

We report for the first time somatic chromosome counts for seven species (*E. calanthum*, *E. cochlidium*, *E. flexuosum*, *E. ibaguense*, *E. myrmecophorum*, *E. puniceoluteum*, and *E. purpureum*) (Table 4). We also confirmed chromosome numbers for *E. denticulatum* and *E. fulgens* and report distinct numbers for *E. radicans*, *E. secundum,* and *E. xanthinum*, which were previously studied by other authors (Table 4). Chromosome numbers range from $2n = 24$ (*E. fulgens*) to $2n = 240$ (*E. cinnabarinum*), indicating extensive variation among species belonging to the subgenus *Amphiglottium*. Extremely high variation within single species was recorded for *E. radicans* ($2n = 40$, 57, 60, 62, and 64); *E. secundum* ($2n = 28$, 40, 48, 52, 68, and 80); and *E. xanthinum* ($2n = 28$, 30, 40, and 60) (Table 4).

Discussion

Both sequence data from the chloroplast DNA *trn*L-*trn*F region and AFLP markers strongly support the monophyly of the taxonomically complex subgenus *Amphiglottium*. The results of this study also suggest the *Tuberculata* clade (subsection *Tuberculata*; Lindley 1852–1859; Table 1) as a monophyletic group, represented in our analyses by *E. cochlidium* and several specimens of *E. secundum* and *E. xanthinum* (Fig. 1). Species belonging to this clade can be recognized by the thick callus in the center of the lip disc, while in the other species the callus is a keel projected longitudinally over the midlobe. Within clade *Tuberculata* there is also strong support for the monophyly of a Brazilian yellow-flowered *E. xanthinum* clade (Fig. 1). The polymorphic *E. secundum* is only recovered as a monophyletic group in the AFLP Fitch MP tree, but this clade has no bootstrap support (Fig. 1a). Brieger (1976–1977) suggested flower color as an important diagnostic character within subsection *Tuberculata*, but the results indicate no phylogenetic signal for this trait, with only the yellow-flowered *E. xanthinum* strongly supported as monophyletic (Figs. 1, 2). PCoA analysis also suggest that specimens of *E. xanthinum* are genetically closely related (Fig. 2).

Subsection *Carinata* (Lindley 1852–1859; Table 1) was not supported as monophyletic by our results. The shape of the callus (a keel projected longitudinally over the midlobe), characteristic of the species of subsection *Carinata*, is clearly plesiomorphic within the subgenus

Amphiglottium, as is the presence of crenate lip margins characteristic of subsections *Carinata* and *Tuberculata*. Instead, both data sets recovered three distinct phylogenetic lineages, all formally included in subsection *Carinata* (Fig. 1): an Andean clade consisting of *E. calanthum*, *E. ibaguense*, and *E. incisum*, which occur in the slopes from the Andean and Guianan ranges; a Brazilian Atlantic clade, with species presenting disjunct distributions along the Brazilian seashore, represented by *E. cinnabarinum*, *E. denticulatum*, *E. puniceolutum*, and *E. fulgens*; and *E. radicans*, with a wide geographical distribution from Mexico to South America, but restricted to high altitudes and open vegetation. The latter is the only morphologically distinct clade of subsection *Carinata*, characterized by the presence of roots along the stem and between the leaves. Apart from distinct biogeographical features, other clades do not present clear morphological diagnostic characters.

The results regarding the monophyly of subsection *Integra* sensu Brieger (1976–1977), represented by *E. myrmecophorum* and *E. purpureum*, are unclear. The *trn*L-*trn*F sequence data and the AFLP Dollo MP tree (Figs. 1, 2) support Brieger's delimitation of subsection *Integra*, morphologically defined by the greenish flowers with brown spots. The diagnostic character of entire lip margins, used by Lindley to define subsection *Integra* is much less restrictive, also including species such as *E. smaragdinum* and *E. campestre*, which are phylogenetically more distant than other species from *Carinata* and *Tuberculata*. On the other hand, the AFLP Fitch MP tree suggests *E. purpureum* to be phylogenetically more closely related to the Atlantic clade, which includes *E. denticulatum*. Interestingly, Miranda (1993) proposed the hypothesis of a hybrid origin of *E. purpureum* from *E. myrmecophorum* and *E. denticulatum* based on the intermediate vegetative and floral morphology of the former. Besides, the two putative parental species occur in sympatry (F. Pinheiro, pers. obs.). The rather distinct chromosome numbers of the putative parents ($2n = 120$ and $2n = 40$; Table 4), however, do not support this hypothesis. Complementary studies are necessary to verify the hybrid origin of *E. purpureum*. The incongruence between AFLP trees may be also due to the inclusion of shared absences of fragments in the Fitch tree, which are not considered by the Dollo criterion (Harris 1999).

Changes in chromosome numbers (autopolyploidization; allopolyploidization) reflect important evolutionary mechanisms, especially in taxonomically complex groups of plants, as they allow genomic rearrangements, stimulate mutation rates, and enable the appearance of novel phenotypes (White 1978). Such mechanisms can facilitate the exploitation of new habitats, contributing to eco-geographical radiation and to the increase in species diversity (Seehausen 2004). Chromosome numbers presented in this study corroborate previous evidence of extreme variability within the subgenus *Amphiglottium* (Tanaka and Kamemoto 1984; Conceição et al. 2006). Extensive variation was observed both between and within species, as for *E. radicans*, *E. secundum*, and *E. xanthinum*. Such extreme variation suggests polyploidy and hybridization as an important role in the diversification of this group. Highly fertile crosses among different species and continuous morphological variability in sympatric populations, as observed for species within the subgenus *Amphiglottium*, may be indirect evidence of hybridization (Rieseberg 1997). In addition, species belonging to the subgenus *Amphiglottium* are well-known for their ability to thrive in new habitats, occupying open areas such as lake beds, steep slopes devoid of vegetation after landslides, roadsides cuts, and sand dunes (Dunsterville 1979; Dressler 1989; Hágsater and Soto Arenas 2005). Indeed, several reports on hybridization among species in the subgenus *Amphiglottium* are available. Dunsterville (1979), Hágsater and Soto Arenas (2005), and Pansarin and Amaral (2008) report the occurrence of hybridization events after high-elevation species have come into contact with another species at lower elevations. Further studies, considering mechanisms of polyploid formation and establishment and the ecological effects of polyploidy in this group, are imperative in order to understand the role of hybridization and polyploidyzation in the diversification of the subgenus *Amphiglottium*.

Conclusions

DNA sequence data and AFLP markers were used to test previous classification systems and investigate patterns of diversification in the subgenus *Amphiglottium*. The results corroborate the monophyly of this group but do not support the current classification system proposed by previous authors. Only subsection *Tuberculata* arises as a highly supported monophyletic group, with subsection *Carinata* being polyphyletic. Results for *Integra* are still obscure and demand further investigation.

Flower morphology in the subgenus *Amphiglottium* was partially informative. The results suggest that the thick callus in the center of the lip disc is a synapomorphy of the clade *Tuberculata*, as possibly are the greenish flowers with brown spots of clade *Integra*. Biogeographical and ecological patterns seem to carry also some strong phylogenetic signal within the subgenus *Amphiglottium*, at least partially, according to the identification of Andean-Guianan and Brazilian Atlantic clades.

This study also confirmed the great variability of chromosome numbers for the subgenus *Amphiglottium*, suggesting polyploidization and hybridization as important mechanisms of speciation, but understanding of speciation

events in this group awaits more detailed cytogenetic studies, such as the use of techniques to detect admixed composition of chromosomes (Hegarty and Hiscock 2005). Complementary studies at species and population levels, as well as data from different molecular markers (i.e., microsatellites) are already being carried out to better understand diversification patterns.

Acknowledgments We thank R. Custódio, J. Leônidas, J. Batista, L.P. Félix, J. Lima-Verde, N. von Atzingen, M. Peixoto, C.N. Fraga, M. Bocayuva, M. Moraes, E.C. Smidt, J. Stancik, G. Lopes, M.A. Farinaccio, and M. Trovó for the help in collecting plant specimens; L.B. Mota, M.M.S. Ferreira, C. Rodrigues, C. Furlan, and S. Blanco for lab assistance; E.L.M. Catharino, R.P. Peres, H. Michelan, and R. Chaves for supporting orchid cultivation at Instituto de Botânica de São Paulo; Naciolinda Obers for help in translating German texts; L.G. Lohman, G.J. Shepherd, E. Hágsater, and M.A. Soto Arenas for valuable comments on previous versions of this manuscript. F.P. and S.K. received fellowships from Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP (03/03063-1 and 06/55121-3, respectively), F.B. received a grant of the Conselho Nacional do Desenvolvimento Científico e Tecnológico, CNPq (303962/2004-6). This study was funded by a grant from FAPESP to FB (03/03062-5).

References

Aceto S, Caputo P, Cozzolino S, Gaudio L, Moretti A (1999) Phylogeny and evolution of *Orchis* and allied genera based on ITS DNA variation: morphological gaps and molecular continuity. Mol Phylogenet Evol 13:67–76

Applied Biosystems (2000) AFLP plant mapping protocol. PE Applied Biosystems, Foster City. Home page at: http://docs.Appliedbiosystems.com. Accessed 10 April 2009

Archibald JK, Mort ME, Crawford DJ, Santos-Guerra A (2006) Evolutionary relationships within recently radiated taxa: comments on methodology and analysis of inter-simple sequence repeat data and other hypervariable, dominant markers. Taxon 55:747–756

Backeljau T, De Bruyn L, De Wolf H, Jordaens K, Van Dongen S, Verhagen R, Winnepenninckx B (1995) Random amplified polymorphic DNA (RAPD) and parsimony methods. Cladistics 11:119–130

Blumenschein A (1960) Número de cromossomas de algumas espécies de orquídeas. Publ Cient Instit Gen, Piracicaba, São Paulo 1:45–50

Borba EL, Shepherd GJ, van den Berg C, Semir J (2002) Floral and vegetative morphometrics of five *Pleurothallis* (Orchidaceae) species: correlation with taxonomy, phylogeny, genetic variability and pollination systems. Ann Bot 90:219–230

Brieger FG (1976–1977) Gattungsreihe Epidendra. In: Brieger FG, Maatsch R, Senghas K (eds) Schlechter Die Orchideen. Paul Parey, Berlin, pp 509–549

Bussell JD, Waycottb M, Chappilla JA (2005) Arbitrarily amplified DNA markers as characters for phylogenetic inference. Perspect Plant Ecol Evol Syst 7:3–26

Carnevali G, Ramírez-Morillo I (2003) *Epidendrum*. In: Berry P, Yatskievych K, Holst B (eds) Flora of the Venezuelan Guayana 7. Missouri Botanical Garden Press, Saint Louis, pp 325–352

Chase M (1999) Molecular systematics, parsimony, and orchid classification. In: Pridgeon AM, Cribb PJ, Chase MW, Rasmussen FN (eds) Genera Orchidacearum 1. Oxford University Press, Oxford, pp 81–88

Cogniaux A (1898–1902) *Epidendrum*. In: Martius CFP, Eichler AG, Urban I (eds) Flora Brasiliensis 3(5). R. Oldenbourg, Munich, pp 30–186

Conceição LP, Oliveira ALPC, Barbosa LV (2006) Characterization of the species Epidendrum cinnabarinum Salzm. (Epidendroideae: Orchidaceae) occurring in Dunas do Abaeté—Salvador, BA—Brasil. Cytologia 71:125–129

Després L, Gielly L, Redoutet B, Taberlet P (2003) Using AFLP to resolve phylogenetic relationships in a morphologically diversified plant species complex when nuclear and chloroplast sequences fail to reveal variability. Mol Phylogenet Evol 27:185–196

Doyle JJ, Doyle JL (1990) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11–15

Dressler RL (1989) Will the real *Epidendrum ibaguense* please stand up? Am Orchid Soc Bull 58:796–800

Dunsterville GC (1979) Orchids of Venezuela—*Epidendrum elongatum*. Am Orchid Soc Bull 48:447–454

Dunsterville GCK, Garay LA (1961) Venezuelan orchids illustrated, vol 2. Andre Deutsch, London

Farris JS (1977) Phylogenetic analysis under Dollo's Law. Syst Zool 26:77–88

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Fitch WM (1971) Toward defining the course of evolution: minimum change for a specific tree topology. Syst Zool 20:406–416

Funk DJ, Omland KE (2003) Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. Annu Rev Ecol Syst 34:397–423

Gower JC (1966) Some distance properties of latent root and vector methods used in multivariate analysis. Biometrika 53:325–338

Guerra M (1983) O uso do Giemsa na citogenética vegetal–comparação entre a coloração simples e o bandeamento. Cienc Cult 35:190–193

Hágsater E (1984) Towards an understanding of the genus *Epidendrum*. In: Tan KW (ed) Proceedings of the 11st World Orchid Conference. 11st World Orchid Conference, Miami, pp 195–201

Hágsater E, Salazar GA (1990) Icones Orchidacearum—orchids of Mexico, part 1. Herbario AMO, Mexico

Hágsater E, Soto Arenas MA (2005) *Epidendrum* L. In: Pridgeon AM, Cribb PJ, Chase MW, Rasmussen FN (eds) Genera Orchidacearum, vol 4. Oxford University Press, Oxford, pp 236–251

Harris SA (1999) Molecular approaches to assessing plant diversity. In: Benson EE (ed) Plant conservation biotechnology. Taylor and Francis, London, pp 11–24

Hegarty MJ, Hiscock SJ (2005) Hybrid speciation in plants: new insights from molecular studies. New Phytol 165:411–423

Lenz LW, Wimber DE (1959) Hybridization and inheritance in orchids. In: Withner CL (ed) The orchids, a scientific survey. Ronald Press, New York, pp 261–314

Lindley J (1852–1859) *Epidendrum*. In: Lindley J (ed) Folia Orchidacea. J. Matthews, London, pp 1–97

Miranda FE (1993) *Epidendrum* × *ormindoi* Miranda hyb. nat. nov. Bradea 6:164–166

Nei M, Li W (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Nat Acad Sci USA 76:5269–5273

Pabst GFJ, Dungs F (1975) Orchidaceae Brasilienses, vol 1. Kurt Schmersow, Hildesheim

Page RDM (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. Comput Appl Biosci 12:357–358

Pansarin ER, Amaral MCE (2008) Reproductive biology and pollination mechanisms of *Epidendrum secundum* (Orchidaceae)—floral variation: a consequence of natural hybridization? Plant Biol 10:211–219

Pinheiro F, Barros F (2006) *Epidendrum puniceoluteum*, uma nova espécie de Orchidaceae do litoral brasileiro. Hoehnea 33:247–250

Pinheiro F, Barros F (2007) *Epidendrum secundum* Jacq. e *E. denticulatum* Barb. Rodr. (Orchidaceae): caracteres úteis para a sua separação. Hoehnea 34:563–570

Pinheiro F, Barros F (2008) Morphometric analysis of *Epidendrum secundum* (Orchidaceae) in southeastern Brazil. Nord J Bot 25:129–136

Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. Bioinformatics 14:817–818

Rieseberg LH (1997) Hybrid origins of plant species. Annu Rev Ecol Syst 28:359–389

Robinson JP, Harris SA (1999) Amplified fragment length polymorphisms and microsatellites: a phylogenetic perspective. In: Gillet EM (ed) Which DNA marker for which purpose? http://webdoc.sub.gwdg.de/ebook/y/1999/whichmarker/index.htm. Accessed 20 March 2009

Seehausen O (2004) Hybridization and adaptive radiation. Trends Ecol Evol 19:198–207

Shepherd GJ (2006) Fitopac versão 1.6.4. Universidade Estadual de Campinas, Campinas

Singer RB, Koehler S (2004) Pollinarium morphology and floral rewards in Brazilian Maxillariinae (Orchidaceae). Ann Bot 93:39–51

Smith A (1994) Rooting molecular trees: problems and strategies. Biol J Linn Soc 51:279–292

Sneath PHA, Sokal RR (1973) Numerical taxonomy: the principles and practice of numerical classification. WH Freeman, San Francisco

Swofford DL (2003) PAUP*: phylogenetic analysis using parsimony (and other methods), version 4.0b10. Sinauer Associates, Sunderland

Swofford DL, Olsen GJ (1990) Phylogeny reconstruction. In: Hillis DM, Moritz C (eds) Molecular systematics. Sinauer Associates, Sunderland, pp 411–501

Taberlet P, Gielly L, Pautou G, Bouvet J (1991) Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Mol Bio 17:1105–1109

Tanaka R, Kamemoto H (1984) Chromosomes in orchids: counting and numbers. In: Arditti J (ed) Orchid biology: reviews and perspectives III. Cornell University Press, Ithaca, pp 324–410

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensibility of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

van den Berg C, Higgins WE, Dressler RL, Whitten WM, Arenas MAS, Culham A, Chase MW (2000) A phylogenetic analysis of Laeliinae (Orchidaceae) based on sequence data from internal transcribed spacers (ITS) of nuclear ribossomal DNA. Lindleyana 15:96–114

van der Pijl L, Dodson CH (1966) Orchid flowers: their pollination and evolution. University of Miami Press, Coral Gables

Vasquez R, Ibisch PL (2004) Orquídeas de Bolívia vol. 2: Subtribus Laeliinae, Polystachinae, Sobraliinae. Editorial FAN, Santa Cruz de la Sierra

Vij SP, Shekhar N (1985) Chromosome number reports LXXXVII. Taxon 34:346–351

White MJD (1978) Modes of speciation. Freeman, San Francisco

Whitten WM, Blanco MA, Williams NH, Koehler S, Carnevali G, Singer RB, Endara L, Neubig KM (2007) Molecular phylogenetics of *Maxillaria* and related genera (Orchidaceae: Cymbidieae) based on combined molecular data sets. Am J Bot 94:1860–1889