Integrating different tools to disentangle species complexes: A case study in *Epidendrum* (Orchidaceae)

Edlley Max Pessoa,[1] Marccus Alves,[1] Anderson Alves-Araújo,[1] Clarisse Palma-Silva[2] & Fábio Pinheiro[2]

1 *Universidade Federal de Pernambuco, Centro de Ciências Biológicas, Departamento de Botânica, 50670-420, Recife, PE, Brazil*
2 *Instituto de Botânica, 04301-902, São Paulo, SP, Brazil*
Author for correspondence: *Fábio Pinheiro, biopinheiro@yahoo.com.br*

Abstract Species delimitation remains a central problem in systematic, taxonomic and evolutionary studies. However, the precise delimitation of species depends on the criteria used to identify lineages and the specific species concept that is applied. Recently, multidisciplinary studies using different data sources have significantly improved the delimitation of species within complex taxonomic groups, leading to an integrative taxonomy. To investigate the species delimitation within the Atlantic clade of *Epidendrum* (subg. *Amphyglottium*), four different species criteria were examined (phenetic differentiation, monophyly, diagnosability, absence of genetic intermediates). Morphometrics, plastid DNA sequences and nuclear microsatellite markers were used to explore the agreement between patterns recovered and species criteria tested. The conflicts among species criteria are discussed in light of pollination ecology, patterns of gene flow, reproductive isolation mechanisms and selective pressures currently acting in deceptive orchid species. Four currently recognized species from the Atlantic clade could be delimitated, including one newly described species, *Epidendrum flammeus*. Three out of five species satisfied the monophyly criterion, and few diagnostic flower characters were found among species. In contrast, nuclear microsatellite data correctly assigned individuals to their respective species, even in the presence of weak reproductive isolation and extensive hybridization events reported in the literature. One important implication of this finding is that phylogenetic studies in *Epidendrum* spp. should make use of single- or low-copy nuclear loci instead of plastid markers, which may be true for other plant groups. The results also indicate that the generalized pollination syndrome found among species of the Atlantic clade, the different levels of gene flow observed between nuclear and plastid markers, and hybridization events are commonly observed as the main evolutionary forces within this orchid group. Finally, we discuss evolutionary processes and taxonomic limits among these species, and we highlight the need to increase the inter-disciplinary approach to investigate species limits in complex plant groups.

Keywords hybridization; orchid evolution; reproductive barriers; speciation; species complex; species delimitation

Supplementary Material Tables S1–S2 and Figures S1–S3 (in the Electronic Supplement) and the alignment are available in the Supplementary Data section of the online version of this article (http://www.ingentaconnect.com/content/iapt/tax).

■ INTRODUCTION

Species delimitation is an old issue with an extensive theoretical and methodological body of literature (de Queiroz, 1998; Sites & Marshall, 2004 and references therein). This subject has recently attracted renewed attention, which is emphasized by the wide interest in barcoding approaches (Lahaye & al., 2008; Hollingsworth & al., 2009; Pires & Marinoni, 2010). Because species are the main units of ecological and evolutionary studies, the identification of boundaries among closely related species, and therefore the inference of the number of extant species, is an essential target of current systematic studies (Duminil & al., 2006; Petit & Excoffier, 2009). To achieve this goal, the cross-validation of species identification using different approaches is desirable (but see Padial & al., 2010), and different tools are available to depict complex morphological and genetic patterns of variation, such as morphometry (Pinheiro & Barros, 2007a, b; Buzzato & al., 2012), and molecular markers (Duminil & al., 2006; Palma-Silva & al., 2011). Delimiting species using multiple and complementary disciplines is called integrative taxonomy (Dayrat, 2005; Padial & al., 2010; Schlick-Steiner & al., 2010).

The perspective that species can be delimited based on multiple lines of evidence has been boosted by the increasing evidence that reproductive barriers are permeable to gene flow (porous genomes, Wu, 2001), that species lineages may diverge despite hybridization and introgression and that a single species can originate polyphyletically by parallel evolution (reviewed in Hausdorf, 2011). Indeed, the general lineage concept of species (de Queiroz, 1998) can be used as a proper conceptual framework related to the use of different types of data to identify species. According to this concept, different patterns of divergence for different types of data and delimitation criteria may arise at different times in the process of speciation (de Queiroz, 1998). For example, different species criteria such as intrinsic reproductive isolation (Mayr, 1942), reciprocal monophyly (de Queiroz & Donoghue, 1988) and the existence of diagnostic characters (Cracraft, 1983) are all properties of evolutionarily distinct lineages. The relative performance of some of these criteria under different evolutionary

scenarios has been investigated, leading to the conclusion that the application of several different criteria should be evaluated considering the relevant forces driving speciation process (reviewed by Padial & al., 2010).

Whereas qualitative traits are relatively easy to measure, the vast majority of phenotypic differences between populations, species, or higher-order taxa are quantitative (Lynch & Walsh, 1998) and will thus require accurate and objective measurement and analysis. The use of multivariate methods to analyze quantitative morphometric data provides the opportunity to summarize large numbers of characters in few discriminant components, allowing the identification of cryptic borders between closely related species (Borba & al., 2002) or the mosaic nature of recombinant hybrids (Lexer & al., 2009). Furthermore, traditional characters used to differentiate species, such as morphology, are increasingly supplemented by approaches based on molecular genetic data. Distinguishing species that have low levels of genetic divergence, either because speciation is recent or because species continue to exchange genes remains a challenging task in evolutionary biology and molecular taxonomy (Duminil & al., 2006; Lexer & al., 2009). The investigation of species limits is greatly facilitated by the availability of molecular markers informative at the species level, from different genomic compartments, most typically nuclear and cytoplasmic genomes (Duminil & al., 2006; Moccia & al., 2007; Palma-Silva & al., 2011). According to Petit & Excoffier (2009) and Hausdorf & Hennig (2010), the use of multiple unlinked nuclear markers experiencing high intraspecific gene flow levels coupled with the use of model-based assignment methods provides the best results to delimit species. Nuclear microsatellites have been pointed out as highly informative markers for interspecific comparisons (Duminil & al., 2006; Hausdorf & Hennig, 2010; Palma-Silva & al., 2011). High cross-transferability among species has been observed for nuclear microsatellites (Barbará & al., 2007; Pinheiro & al., 2009b), and the occurrence of homoplasy is overcome by the use of multiple unlinked loci (Duminil & Di Michele, 2009).

Our study group, *Epidendrum* L. (Orchidaceae), is the largest (1500 species) and most widespread (Southern United States to North Argentina) Neotropical orchid genus (Hágsater & Soto-Arenas, 2005). *Epidendrum* is renowned for uncertainties regarding delimitation within species complexes, because

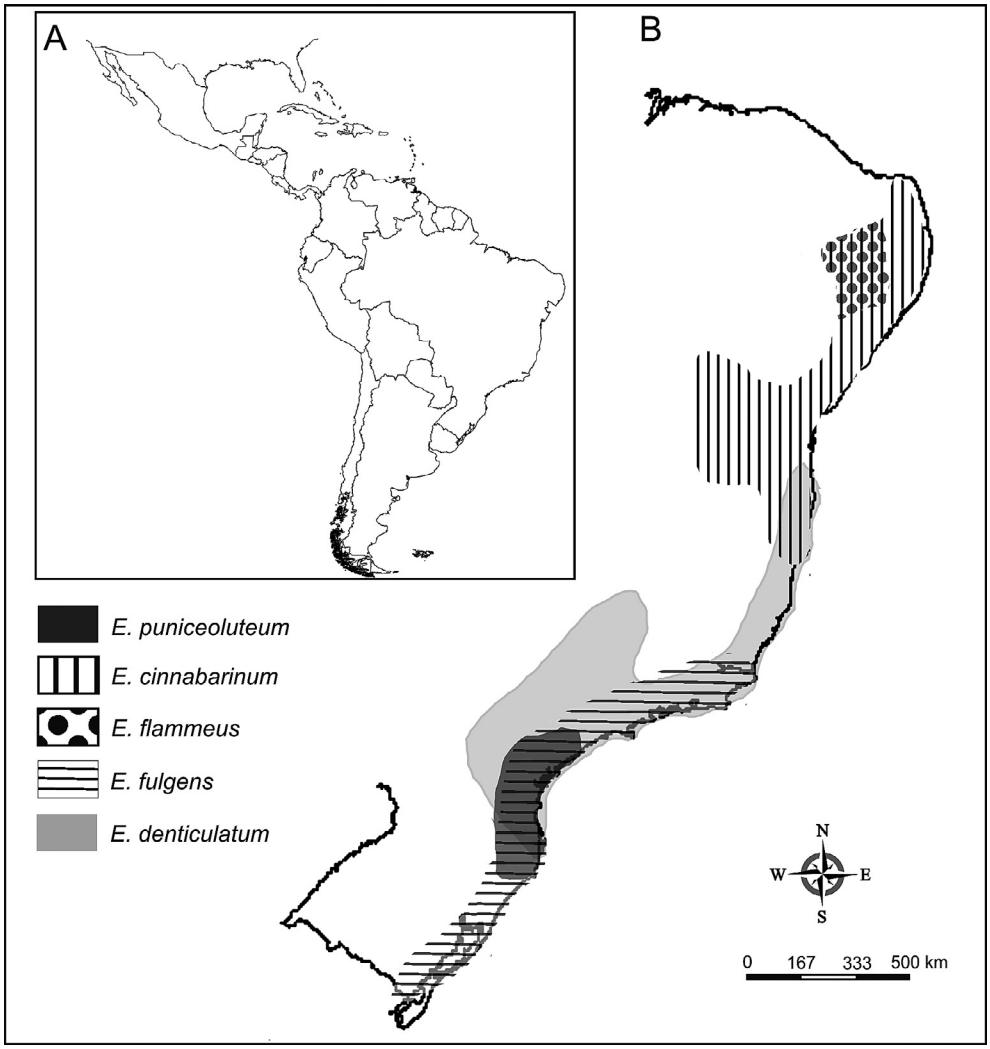

Fig. 1. Geographical distribution of *Epidendrum* species of the Atlantic clade. **A,** Latin American map; **B,** detail of Brazilian Atlantic coast.

many species show impressive morphological variation. Studies within the genus are primarily limited to the description of new species based on qualitative morphological data, and complementary taxonomic and evolutionary studies are comparatively rare (see review by Hágsater & Soto-Arenas, 2005). Blurred morphological boundaries between many closely related species are common within the genus, and detailed data describing patterns of intraspecific morphological variation are restricted to few species (Pinheiro & Barros, 2007a, b). Recently, a highly informative set of nuclear (Pinheiro & al., 2008a, b) and plastid markers (Pinheiro & al., 2009c) was developed for *Epidendrum*, which contributed substantially to the investigation of evolutionary processes at low taxonomic levels within the genus (Pinheiro & al., 2010, 2011).

Species of *Epidendrum* subg. *Amphyglottium* (Salisb.) Brieger were first recognized as a monophyletic group by Hágsater & Soto-Arenas (2005). Recently, Pinheiro & al. (2009a) expanded the previous phylogenetic study of Hágsater & Soto-Arenas (2005) by analyzing 14 species. Strongly supported clades representing distinct biogeographical regions were recovered, such as the Andean clade, with species mainly distributed along the Andean and Guianan mountain ranges, and the Atlantic clade, which includes species distributed along the Brazilian coast, in the Cerrado and Caatinga vegetation domains (Fig. 1). The Atlantic clade comprises four species (*Epidendrum cinnabarinum* Salzm. ex Lindl., *E. denticulatum* Barb. Rodr., *E. fulgens* Brongn. and *E. puniceoluteum* Pinheiro & Barros) (Fig. S1 in the Electronic Supplement). Extensive variation of morphological traits, allopatric vs. sympatric/parapatric occurrence and wide geographic distribution offer interesting possibilities for investigating species limits, reproductive isolation and phylogeography within this group. Hybridization and late generation introgression between *E. fulgens* and *E. puniceoluteum* were identified using nuclear and plastid microsatellites (Pinheiro & al., 2010), which suggests an important role of interspecific gene flow in generating morphological and chromosome number diversification. Moreover, pure parental individuals of both species and hybrid individuals were identified with high confidence using Bayesian assignment analysis, in agreement with previous observations based on morphological characters (Pinheiro & Barros, 2006). Even within the geographical range of *E. fulgens* it was possible to identify important phylogeographic breaks and historical demographic events (gene flow, bottleneck) that shaped the current genetic patterns observed in natural populations (Pinheiro & al., 2011).

To explore species limits and speciation hypotheses among closely related species, the general lineage concept of species was used as the conceptual framework to test the utility of different species criteria, in the context of integrative taxonomy. The following four criteria for species delimitation, based on morphological and DNA polymorphism, were tested: (1) phenetic differentiation (Sokal & Crovello, 1970); (2) reciprocal monophyly (de Queiroz & Donoghue, 1988); (3) diagnosability, which refers to the appearance of fixed differences (Cracraft, 1983); and (4) genotypic clusters recognized by a deficit of genetic intermediates (Mallet, 1995). Furthermore, the existence of a new species was also tested in our study, described below as *E. flammeus*. Qualitative morphological characters, morphometric data and multiple nuclear and plastid markers clearly indicate species limits within the Atlantic clade, clarifying evolutionary and speciation processes within this group. The cross-validation of species identification using different tools, following the general lineage species concept and an integrative taxonomic approach, proved to be useful for depicting complex evolutionary relationships that are commonly found among many plant groups.

■ MATERIALS AND METHODS

Group of interest. — All species of the Atlantic clade were analyzed in this study. *Epidendrum flammeus* occurs within the geographical domain of the Atlantic clade, and shares many morphological characters with *E. fulgens* and *E. denticulatum*. All of the specimens of *E. flammeus* were collected in one granitic rock outcrop within the Borborema mountain range, in northeastern Brazil. Samples of *E. flammeus* were found during recent fieldwork conducted to make floristic inventories of rock outcrops in the Serra da Borborema massif, within Caatinga. Preliminary qualitative morphological comparisons with other specimens of subg. *Amphyglottium* indicated that these collections might represent a new species. Additional herbarium material was also found from close geographic localities, identified as *E. fulgens* (see commentaries in species description). For this reason, samples of *E. flammeus* were included in this study and its status as a new species was investigated. Population origin and sample sizes are described in Table 1.

Morphometric analysis. — To quantify continuous patterns of morphological variation among species of the Atlantic clade (*E. cinnabarinum*, *E. denticulatum*, *E. fulgens*, *E. puniceoluteum*) and the new species described, variation in 16 flower traits (Table S1 in the Electronic Supplement) was measured in 420 individuals, using a digital ruler and following the procedure described in Pinheiro & Barros (2007a). Discriminant function analysis (DA) was used to assess multivariate morphological differentiation among populations. Wilks' Lambda, jackknifed classification, which assigns unclassified specimens to groups, and F-to remove statistics, which provide an indication of the relative importance of each variable, are also reported. Mean and standard deviation values for each character and each species were calculated, and are depicted in Box-plot graphics. Data were analysed and tested for multivariate normality using SYSTAT v.11.0. (SYSTAT Software, Richmond, California, U.S.A.).

Molecular phylogenetic study. — A recent molecular phylogenetic study of subg. *Amphyglottium* (Pinheiro & al., 2009a) was used as the basic framework to test the phylogenetic position of *E. flammeus*. The precise phylogenetic relationships of the new species were then determined by sequencing three plastid regions, *trnT-trnL*, *trnL-trnF* and *rpl32-trnL* for all species, using primers described by Shaw & al. (2005, 2007). In total, 20 individuals were sequenced, representing 10 previously described species of subg. *Amphyglottium*, plus the newly

described *E. flammeus*. *Epidendrum campestre* Lindl. and *E. nocturnum* Jacq. were used as outgroups. DNA extraction, amplification reactions and plastid DNA sequencing were carried out following Pinheiro & al. (2009c). All of the sequences used in this study were submitted to GenBank (Appendix). A total of 20 terminal operational taxonomic units (OTUs) were selected for this study, and five of them were represented by more than one accession each (*Epidendrum cinnabarinum*, *E. fulgens*, *E. puniceoluteum*, *E. denticulatum*, *E. flammeus*).

Each sequence accession of the *trnL-trnF*, *rpl32-trnL* and *trnL-trnT* regions was aligned with Clustal W option in the BioEdit Sequence Alignment Editor (Hall, 1999). The resulting automated alignment was manually edited in BioEdit v.5.0.6 and then was exported as a Nexus file for maximum parsimony (MP) and maximum likelihood (ML) analyses. The MP analysis was performed following Pinheiro & al. (2009a), using the criterion of Fitch (1971), excluding uninformative characters, and with ACCTRAN optimization. Ten thousand addition sequence replicates were performed by stepwise addition and holding 10 trees per replicate, and TBR branch swapping on best trees. The MP analysis was performed with PAUP* v.4.0b10 (Swofford, 2002). The ML analysis was conducted using RAxML v.7.0.4 (Stamatakis, 2006) and RAxML-GUI v.1.1 (Silvestro & Michalak, 2011). The GTR+Γ substitution model, which allows rate variation among sites, was determined using jModeltest v.0.1.1 (Posada, 2008) under the Akaike

Table 1. *Epidendrum* species and populations analyzed in this study. Sample sizes for the morphometric analysis, nuclear microsatellite markers (SSR) assays for assignment tests and plastid intergenic spacer sequences (CP) are indicated.

		Species	Population	Sample Size		
				Morphometry	SSR	CP
Epidendrum subg. *Amphyglottium*	Atlantic clade	*E. cinnabarinum* Salzm. ex Lindl.	Pirambu	25	–	1
			Lagoa do Abaeté	13	–	1
		E. denticulatum Barb. Rodr.	Olivença	9	20	–
			Alcobaça	11	–	–
			Poços de Caldas	5	–	–
			Marambaia	10	20	1
			Botucatu	13	–	–
			São Paulo	22	–	–
			Itapeva	9	–	1
		E. flammeus E. Pessoa & M. Alves	Pedra do Cachorro	51	53	4
		E. fulgens Brongn.	Parati	22	–	1
			Bertioga	26	20	–
			Cananéia	30	–	–
			Itajaí	26	–	–
			Florianópolis	33	–	–
			Imbituba	32	20	1
			Porto Alegre	17	–	–
		E. puniceoluteum Pinheiro & Barros	Imbituba	13	20	1
			Cananéia	26	–	–
			Pontal do Sul	27	20	1
		E. calanthum Rchb. f. & Warsz.	Serra Pacaraima	–	–	1
		E. macrocarpum Rich.	Recife	–	–	1
		E. myrmecophorum Barb. Rodr.	Araruama	–	–	1
		E. radicans Pav. ex Lindl.	Oaxaca	–	–	1
		E. secundum Jacq.	Serra do Rio do Rastro	–	–	1
		E. xanthinum Lindl.	Santa Bárbara	–	–	1
Outgroup		*E. campestre* Lindl.	Santana do Riacho	–	–	1
		E. nocturnum Jacq.	Cananéia	–	–	1
		Total		420	173	20

information criterion (AIC). To find the optimal likelihood tree, we ran 100 independent tree searches on the combined matrix. The support for individual branches was evaluated using non-parametric bootstrapping (Felsenstein, 1985) with 1000 thorough bootstrap replicates. The categories of bootstrap support (BS) considered were unsupported (< 50%), weak (50%–74%), moderate (75%–84%), and strong (85%–100%).

Nuclear markers and assignment tests. — Eleven nuclear microsatellite markers were used in this study, with seven isolated from *E. fulgens* (markers Eff06, Eff26, Eff43, Eff45, Eff61 and Eff70, Pinheiro & al., 2008a; Eff48, primer forward 5′-TGACCGTTTGAACCTTTTGGT-3′, reverse 5′-ATCCAG GCATGAGCAGCA-3′) and four isolated from *E. puniceoluteum* (markers Epp18, Epp49, Epp86 and Epp96, Pinheiro & al., 2008b). In total, 173 individuals of species of the Atlantic clade including *E. flammeus* were genotyped. In order to exclude the possibility of genetic differentiation by genetic drift and low gene flow, two populations of each species were analysed, excluding *E. flammeus*, from which samples were available only from one locality (see Table 1 for details). *Epidendrum cinnabarinum* is a polyploid species with $2n = 240$, and it was not included in this analysis because of problems in genotyping assays (loci showing more than two alleles across almost all individuals). Amplification reactions and genotyping procedures followed Pinheiro & al. (2008b).

Based on simulations performed by Hausdorf & Hennig (2010), two assignment methods were chosen to classify individuals based on multiple nuclear loci. The first method is implemented in STRUCTURE v.2.3.3 (Hubisz & al., 2009), which assigns individuals to genetic clusters (K) and estimates the admixture proportions (Q) for each individual. A set of models was chosen in which individuals have admixed ancestries and correlated allele frequencies. The number of genetic clusters (K) was set from a minimum of 1 to a maximum of 10, and replicate runs were performed for each K-value with a burn-in of 250,000 and 1 million iterations each. To define the most probable number of K present in the data, we used the ad hoc criteria $L(K)$ and ΔK proposed by Pritchard & al. (2010) and Evanno & al. (2005), calculated in STRUCTURE HARVESTER v.6.0 (Earl & Holdt, 2011). STRUCTURAMA v.2.0 was also used to estimate the number of discrete genetic clusters (Huelsenbeck & Andolfatto, 2007). In contrast to STRUCTURE, in STRUCTURAMA, the number of clusters and the assignment of individuals to clusters can both be considered random variables that follow a Dirichlet process prior in this Bayesian approach (Huelsenbeck & Andolfatto, 2007). Multiple analyses were run to explore whether the results remained consistent. All of the Markov chain Monte Carlo analyses were run for 1 million generations with a sample frequency of 100, and the first 4000 samples were discarded as burn-in.

Population aggregation analysis. — To test whether named species were diagnosable and consistently different from each other, population aggregation analysis (PAA; Davis & Nixon, 1992) was applied to the species of the Atlantic clade. PAA is a method for delimiting species via the search for fixed character differences among local populations or groups of populations. Following Reeves & Richards (2011), this method was applied to search for fixed alleles, morphometric characters and qualitative features that are exclusive to each species analyzed. Fixed alleles among species, based on nuclear microsatellite data, were determined considering two populations per species, excluding *E. flammeus* (only one population) and *E. cinnabarinum* (not analyzed).

■ RESULTS

The multivariate normality was confirmed for all datasets ($P > 0.01$ for all comparisons). The ordination diagram of the DA indicates a clear separation among the species, considering the first three canonical axes (Fig. 2, Wilks' Lambda = 0.0008, $P < 0.0001$) which represent 97.5% of the total sample variation. The five most discriminant variables in the model are length of the callus of the lip, column length, length and width of the central lobe of the lip, and lip length. The jackknifed classification matrix produced by DA shows an average of 98% of correct classification of individuals into the previously assigned species, ranging from 100% correct classification in *E. fulgens* and *E. cinnabarinum* to 94% in *E. flammeus* (Table S2 in the Electronic Supplement). The pattern of variation of the above characters is represented by box-plots (Fig. S2 in the Electronic Supplement). High intraspecific variability is observed for almost all of the characters, which overlap among most species. Only *E. cinnabarinum* shows clear discontinuities, mainly in the length of the dorsal and lateral sepals, the petal and the callus of the lip (Fig. S2).

The *trnL-trnF* gene sequences were 1040 bp long, *rpl32-trnL* 832 bp, and *trnL-trnT* 683 bp, excluding flanking regions, insertions and ambiguously aligned fragments. The combined molecular matrix consisted of 2557 aligned bp from the 20 terminal OTUs with nucleotide frequencies of: A = 0.3855, C = 0.1258, G = 0.1462, and T = 0.3425, with a transition/transversion ratio = 2 (kappa = 4.9217825).

The topologies of MP and ML trees recovered in this study were identical, and for this reason only results from the ML analysis are shown (Fig. 3). The main difference observed, in comparison to the study of Pinheiro & al. (2009a), was the position of *E. radicans* nested within the Andean clade (Fig. 3). Subgenus *Amphyglottium* is supported as a monophyletic group (BS 100; Fig. 3). Strong support (BS ≥ 85) was observed for both the Atlantic clade and subsect. *Tuberculata*. In the Andean clade *E. radicans* is sister to the other species, *E. calanthum* and *E. macrocarpum*. Within the Atlantic clade, *E. cinnabarinum* is sister to the other species with BS = 98. Both *E. denticulatum* and *E. flammeus* are monophyletic, with bootstrap values of 90% and 92%, respectively. In contrast to the results obtained by Pinheiro & al. (2009a) using AFLP, *E. fulgens* and *E. puniceoluteum* were not monophyletic (Fig. 3).

Bayesian assignment results obtained by STRUCTURE and STRUCTURAMA correctly assigned all individuals to previously recognized species. The statistic $L(K)$ proposed by Pritchard & al. (2010) and the ad hoc criterion ΔK proposed by Evanno & al. (2005) indicated $K = 4$ (Table 2; Fig. S3 in the Electronic Supplement). Results recovered by posterior

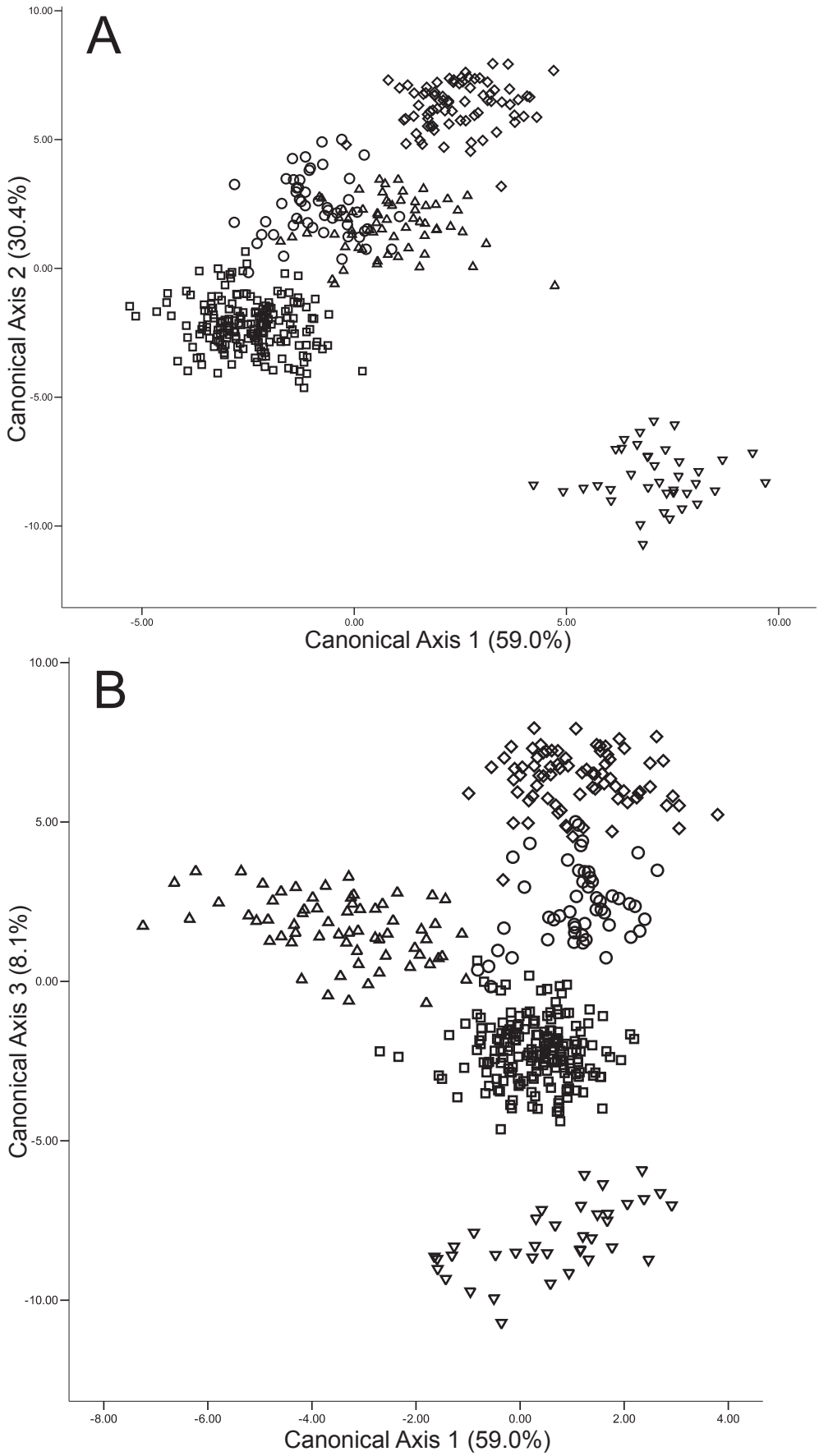

Fig. 2. Discriminant scatter plots showing morphological similarities among *E. cinnabarinum* (▽), *E. denticulatum* (◇), *E. flammeus* (○), *E. fulgens* (□) and *E. puniceoluteum* (△). **A,** first and second axes represent 89.4% of the variation of the dataset; **B,** first and third axes represent 67.1% of the total variation among samples.

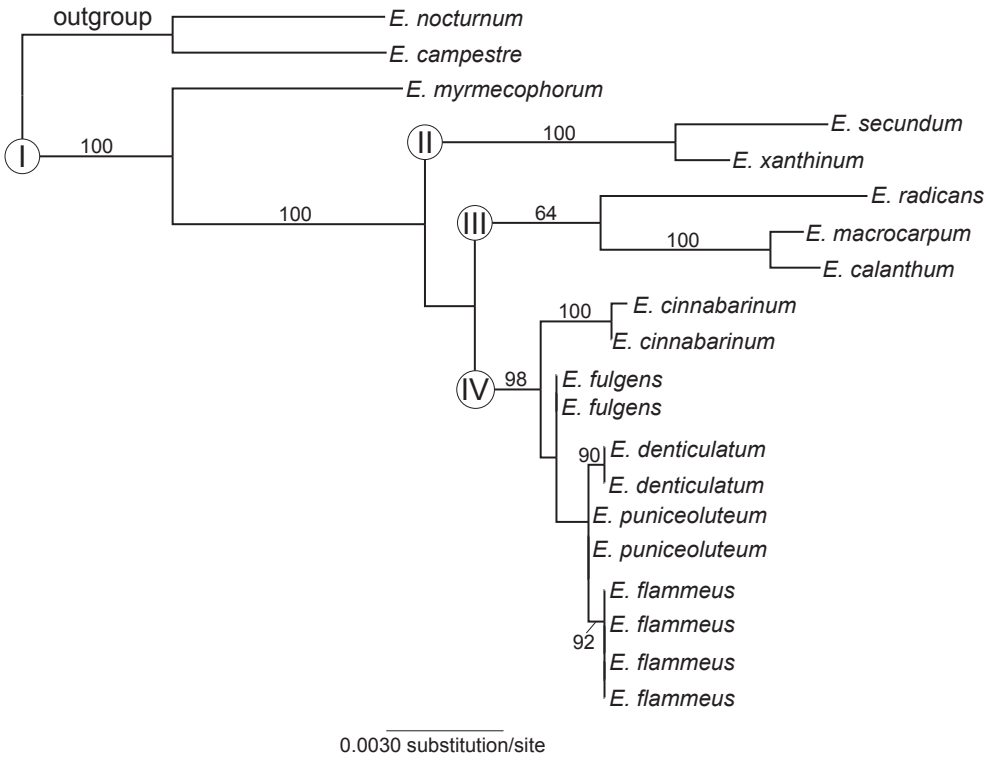

Fig. 3. Maximum-likelihood phylogram of *Epidendrum* subg. *Amphyglottium*, including *E. campestre* and *E. nocturnum* as outgroups, based on combined *trnT-trnL*, *trnL-trnF* and *rpl32-trnL* data. The phylogram shown is based on the best-scoring maximum-likelihood tree. Clades recognized in this study are indicated as follows: (I) *E.* subg. *Amphyglottium*, (II) *E.* subsect. *Tuberculata*, (III) Andean clade, (IV) Atlantic clade. Maximum likelihood bootstrap support values above 50% are indicated on branches.

probabilities calculated by STRUCTURAMA also showed $K = 4$ as the most probable number of genetic clusters (Table 2). Assignment probabilities were higher than 0.9 for almost all individuals, indicating a clear clustering and no indication of admixture for almost all specimens (Fig. 4). Only individuals of *E. puniceoluteum* showed signs of admixture with *E. fulgens*.

The eleven nuclear microsatellite loci analyzed revealed a total of 109 alleles among species of the Atlantic clade (excluding *E. cinnabarinum*). Of those, PAA identified 49 private alleles distributed among *E. denticulatum*, *E. flammeus*, *E. fulgens* and *E. puniceoluteum*. The number of diagnostic alleles ranged from 16 in *E. denticulatum* and *E. fulgens* to 7 in *E. flammeus* (Table 3). Diagnostic characters were also found for both quantitative and qualitative morphological traits. Four exclusive morphological characters were found in *E. cinnabarinum*, two in *E. denticulatum* and one in *E. fulgens*. Diagnostic morphological characters were not found in *E. flammeus* and *E. puniceoluteum* (Table 3).

■ DISCUSSION

When combining different data types to test hypotheses about lineage diversification, authors go beyond the naming of species by reconstructing the evolutionary processes involved in their origin (Schlick-Steiner & al., 2010). The characterization of evolutionary processes underlying lineage diversification has dramatically changed the way that species are recognized (Mayr, 1942). As an example, an extensive number of

Table 2. Estimates of cluster number (K) from STRUCTURE and STRUCTURAMA analyses using nine microsatellite loci for three datasets, including *E. flammeus* and *E. denticulatum*, *E. flammeus* and *E. fulgens*, and *E. flammeus* and *E. puniceoluteum*. STRUCTURE ad hoc statistics $L(K)$ and ΔK and STRUCTURAMA posterior probability distributions $E(K)$ are given.

No. of populations (K)	STRUCTURE ad hoc statistics		STRUCTURAMA posterior probability distributions
	$L(K)$	ΔK	$E(K)$
1	−4620.73	–	0.00
2	−3781.25	8.40	0.00
3	−3423.44	0.77	0.01
4	**−3103.32**	**32.72**	**0.43**
5	−3041.53	8.24	0.37
6	−3067.23	0.41	0.14
7	−3068.70	0.25	0.03
8	−3091.25	1.06	0.01
9	−3230.99	0.74	0.00
10	−3125.18	–	–

The $L(K)$ value in bold points at the beginning of similar estimates obtained by log Pr(X|K), and indicates the correct number of groups ($K = 4$) according to Pritchard & al. (2010). The highest ΔK value estimated from STRUCTURE ($K = 4$) and the highest posterior probability estimated from STRUCTURAMA ($K = 4$) are shown in bold.

cryptic species have been described, and the number of species has decreased through the demonstration of conspecificity of nominal taxa (Schlick-Steiner & al., 2010). Through its interdisciplinary approach, in which phylogeography, systematics, population genetics, ecology and morphology provide the necessary framework for comparative analyses of biodiversity, integrative taxonomy should have a deep impact across all levels of biological organization (Dayrat, 2005; Hendry & al., 2010). Given that the Neotropics harbour an impressive number of species, and a still unknown number of species remain to be described, the integration of several disciplines should be especially helpful in this region (Diniz-Filho & al., 2008; Pires & Marinoni, 2010; Antonelli & Sanmartín, 2011).

Integrative taxonomy has included new concepts and methods in order to delimit species, but a lack of consensus about how data from different sources should be integrated is still evident (Padial & al., 2010). Congruence among different sources of data strengthens species delimitation in most cases (Savolainen & al., 2006; Moccia & al., 2007; Reeves & Richards, 2011). This approach, named integration by congruence (Padial & al., 2010), tends to promote taxonomic stability, since most taxonomists will share similar opinions on the validity of a species supported by several datasets. However, recent radiations may often be overlooked under this point of view (Padial & al., 2010; Barrett & Freudenstein, 2011). On the other hand, the heterogeneous nature of evolutionary forces often precludes full character congruence in species or complete reproductive isolation, and such conflicts could indicate ongoing speciation (Borba & al., 2002; Pillon & al., 2009; Palma-Silva & al., 2011; Duminil & Di Michele, 2009). Because different species concepts often refer to different stages during speciation, full congruence among criteria will be achieved only in a late stage of differentiation (de Queiroz, 1998). In such situations, species recognition could be based on a single dataset, following the framework of integration by cumulation (Padial & al., 2010). A major advantage of this approach is that species which originated from recent radiations could be easily delimited (Padial & al., 2010). However, an overestimation in species number is expected when only a single line of evidence is considered (Padial & al., 2010).

The contrasting approaches of integration by congruence and integration by cumulation need to be coordinated taking into account the evolutionary forces associated leading to speciation in the taxonomic group of interest (Hendry & al., 2010; Padial & al., 2010). Since the concordance among data types is not strictly necessary for justifying the existence of

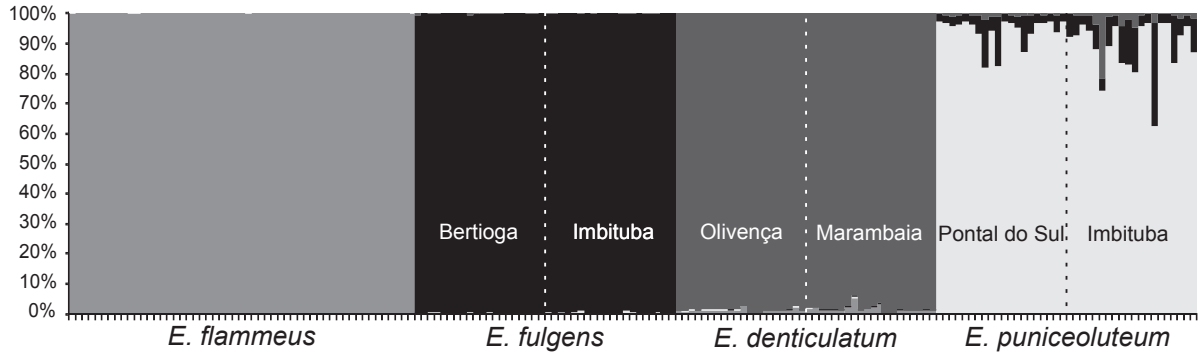

Fig. 4. Maximum posterior assignment probabilities for 173 specimens of *E. flammeus* (Pedra do Cachorro population), *E. fulgens* (Bertioga and Imbituba populations), *E. denticulatum* (Olivença and Marambaia populations) and *E. puniceoluteum* (Imbituba and Pontal do Sul populations), analyzed with STRUCTURE with $K = 4$. Each vertical bar represents an individual. The proportion of color in each bar represents an individual's assignment probability to different species. Vertical dotted lines delimit different populations within species. See Table 1 for population details.

Table 3. Number of fixed (private) alleles and presence (1) and absence (0) of diagnostic morphological characters among *E. cinnabarinum*, *E. denticulatum*, *E. flammeus*, *E. fulgens* and *E. puniceoluteum* detected by PAA.

Character	*E. cinnabarinum*	*E. denticulatum*	*E. flammeus*	*E. fulgens*	*E. puniceoluteum*
Fixed microsatellite alleles	–	16	7	16	10
Lip with brown dots	0	0	0	1	0
Flowers pink	0	1	0	0	0
Lip callus white	0	1	0	0	0
Dorsal sepal >18 mm long	1	0	0	0	0
Mid-lobe of lip entire	1	0	0	0	0
Callus of lip >7 mm long	1	0	0	0	0
Petal >18 mm long	1	0	0	0	0

distinct species (de Queiroz, 1998; Padial & al., 2010; Barrett & Freudenstein, 2011), we adopted the cumulative integration of multiple lines of evidence as a criterion to combine the different data sources investigated in this study. Furthermore, species delimitation will be discussed in the light of evolutionary trends (Padial & al., 2010), based mainly on specific ecological and genetic characteristics observed in subg. *Amphyglottium*.

The phylogeny recovered in this study shows a topology similar to the tree published by Pinheiro & al. (2009a). Within the Atlantic clade, support for the monophyly criterion (de Queiroz & Donoghue, 1988) is observed only for *E. cinnabarinum*, *E. denticulatum* and *E. flammeus* (Table 4). *Epidendrum fulgens* and *E. puniceoluteum* are not monophyletic in this clade. Thus, although monophyly is a proper criterion for species delimitation under the general lineage concept, monophyletic groups might not be detected in the face of gene flow and incomplete lineage sorting, factors that are common at lower taxonomic levels (Knowles & Carstens, 2007). *Epidendrum fulgens* occurs in sympatry with three other species (*E. denticulatum*, *E. puniceoluteum*, *E. secundum*), and hybridization events are known to occur among them (Pinheiro & al., in prep.). In fact, hybridization and introgression between *E. fulgens* and *E. puniceoluteum* in six sympatric populations were detected by nuclear and plastid microsatellites (Pinheiro & al., 2010). Indeed, reproductive isolation mechanisms are not complete among the species of the Atlantic clade (Pansarin & Amaral, 2008; Pinheiro & al., 2010; Pinheiro, unpub.), and support for the reproductive isolation criterion (Mayr, 1942) was observed only for *E. cinnabarinum*. Moreover, shared haplotypes were detected by Pinheiro & al. (2010) in some individuals, suggesting late generation backcrossing, which can mislead results from phylogenetic trees. However, the monophyly observed for *E. cinnabarinum*, *E. denticulatum*, and *E. flammeus* suggests the absence of haplotype sharing caused by late-generation introgression, indicating the existence of strong postzygotic barriers, as F1 hybrids can be easily produced in crossing experiments (Pinheiro, unpub.). Further studies investigating the genetic structure of other hybrid zones in the range of species of the Atlantic clade will help to clarify the strength of reproductive barriers and species cohesion within this group.

Although plastid markers did not support a pattern of monophyly for all species, nuclear microsatellite markers correctly assigned all individuals to the known species (Fig. S3; Fig. 4), and genetic intermediates were rarely found (*E. fulgens* × *E. puniceoluteum*, Fig. 4), strongly supporting the genetic intermediates criteria (Table 4) (Mallet, 1995). The different levels of resolution recovered by plastid and nuclear markers could result from differential rates of gene flow between the two genomes. According to Petit & Excoffier (2009), genomic compartments with high levels of intraspecific gene flow are predicted to experience less introgression (interspecific gene flow), because genetic drift reduces the probability of an introgressed allele to increase in frequency by chance. In plants pollen movement is often the predominant form of gene flow (reviewed in Duminil & al., 2007). Given that observation, nuclear markers are less prone to introgression and are thus more appropriate to delimit species (Petit & Excoffier, 2009).

Table 4. Conformance of *Epidendrum* species of the Atlantic clade with four criteria for species delimitation.

Species	Phenetic	Monophyly	Diagnosability	Genotypic cluster
E. cinnabarinum	Yes	Yes	Yes	Yes
E. denticulatum	Yes	Yes	Yes	Yes
E. flammeus	Yes	Yes	Yes	Yes
E. fulgens	Yes	No	Yes	Yes
E. puniceoluteum	Yes	No	Yes	Yes

Recent empirical data show that seeds have poorer dispersibility than pollen, which could explain the higher levels of introgression observed for maternally inherited plastid DNA (Du & al., 2009; Palma-Silva & al., 2011). In fact, Pinheiro & al. (2010, 2011) found that gene flow via pollen in *Epidendrum* is more than ten-fold higher than that via seeds. Consequently, even in the presence of hybridization and introgression (Pinheiro & al., 2010), nuclear markers are less introgressed because they experience high levels of gene flow. These results strongly agree with patterns that have been observed in many other angiosperms (reviewed in Petit & al., 2005; Duminil & al., 2007), and suggest that nuclear markers coupled with multilocus assignment methods (e.g., Duminil & al., 2006; Hausdorf & Hennig, 2010) represent the best option for species delimitation. However, additional analyses including additional populations may be required to demonstrate that species show deep genetic structuring among populations, which might result in an overestimation of the number of true species (Pritchard & al., 2010). One important implication of this finding is that phylogenetic studies in *Epidendrum* spp. should make use of single- or low-copy nuclear loci instead of plastid markers, which might also be true for other plant groups.

Few morphological diagnostic characters supporting both the phenetic (Sokal & Crovello, 1970) and diagnosability (Cracraft, 1983) criteria were found among species of the Atlantic clade (Table 3). Four of seven morphological characters were restricted to *E. cinnabarinum* (Table 3; Fig. S2), which have larger flowers and are visited by hummingbirds (Pinheiro, unpub.), in contrast to the other species, which have smaller flowers and are mainly pollinated by butterflies (Almeida & Figueiredo, 2003; Pansarin & Amaral, 2008; Fuhro & al., 2010). *Epidendrum denticulatum* showed two diagnostic morphological characters, *E. fulgens* one, and no exclusive flower trait could be found for either *E. flammeus* or *E. puniceoluteum*. The morphological delimitation of *E. puniceoluteum* (Pinheiro & Barros, 2006) and *E. flammeus* (this study) was possible only when considering the combination of multiple morphometric characters (Fig. 2; Table S1). Usually, *Epidendrum* species of subsect. *Amphyglottium* lack strong premating barriers because many species share an extensive number of pollinator species (Byerzychudek, 1981; Almeida & Figueiredo, 2003; Pansarin & Amaral, 2008; Hágsater & Soto-Arenas, 2005). The overall flower similarities and the absence of diagnostic characters are more related to convergence to butterfly pollination systems in species of subg. *Amphyglottium* and, more specifically, of

the Atlantic clade. In general, plants with generalist pollination systems might be less likely to experience strong directional selection on floral traits (Waser, 1998), and diagnostic flower characters could be less evident in such species (Pellegrino & al., 2005).

The relaxed selection related to pollinator behavior could be responsible for high levels of intraspecific morphological variation (Juillet & Scopece, 2010). In food-deceptive orchids, outcrossing prevails because pollinators avoid plants in the same patch, promoting gene flow by pollen over long distances and reducing the chances of geitonogamous pollination (Cozzolino & Widmer, 2005). This behavior is likely to result in low stabilizing selection for floral traits in deceptive species, and thus floral polymorphisms arising from mutations can accumulate and increase intraspecific variation in natural populations (Juillet & Scopece, 2010). According to Pinheiro & al. (2010), habitat selection contributes to species cohesion as a potential post-mating, postzygotic barrier that limits gene flow. Future studies should include long-term field experiments, including reciprocal transplants and reproductive manipulations, to provide direct tests of this hypothesis.

According to the results obtained in this study, species of the Atlantic clade satisfy most of the species criteria examined (Table 4), and thus merit recognition as distinct species. All of the species satisfy at least three of the four species criteria investigated: (1) phenetic differentiation based on morphological discontinuities observed on morphometric results (Sokal & Crovello, 1970); (2) monophyly (de Queiroz & Donoghue, 1988); (3) diagnosability (Cracraft, 1983), which was found in both molecular and morphological traits; and (4) genotypic clusters recognized by a deficit of genetic intermediates (Mallet, 1995), as shown by nuclear microsatellite data. The multidisciplinary approach adopted here to investigate species limits in this orchid group allowed for the delimitation of closely related species of the Atlantic clade and the recognition of a new species, *E. flammeus*. The new species is described and illustrated below. An identification key for species from the Atlantic clade is also provided. Species of subg. *Amphyglottium* were used as models to understand diversification patterns in *Epidendrum*. The huge number of species in this genus is an intrinsic impediment for multidisciplinary studies, but different studies focusing on specific and smaller *Epidendrum* groups should be a suitable approach to understand evolutionary patterns within this genus. Moreover, future studies should consider the use of multiple lines of evidence to investigate species limits in different plant groups, in order to depict evolutionary trends and speciation processes according to an integrative taxonomic approach.

■ DESCRIPTION OF THE NEW SPECIES

Epidendrum flammeus E. Pessoa & M. Alves, **sp. nov.** – Type: Brazil. Pernambuco, Município de São Caetano, RPPN Pedra do Cachorro, 08°14′11.6″ S, 36°11′31.9″ W, 1032 m elev., 7 Mar 2010, *E. Pessoa & K. Mendes* 306 (holotype: UFP; isotypes: NY, RB, SP). — Figure 5.

Epidendrum flammeus is morphologically related to *E. denticulatum*, but differs by having yellow, orange or red flowers, these with a curved lip.

Description. – Rupicolous, caespitose herb. Stems 10.5–73.0 cm long, 0.5–1.0 cm wide, simple, cane-like, terete. Leaves 3.0–9.0 cm long, 0.8–3.0 cm wide, distichous, distributed throughout the stems, coriaceous, lanceolate to elliptic, apex rounded, slightly emarginate, margin entire. Inflorescence terminal, racemose; peduncule 14.0–77.0 cm long, covered by acute, amplexcaulous sheaths; rachis of inflorescence 3.0–4.0 cm long. Floral bracts 0.3–0.9 cm long, 0.1–0.2 cm wide, much shorter than ovary, triangular, lanceolate, apex acute. Flowers 5–26, simultaneous, nonresupinate, yellow, orange or red. Ovary pedicellate, 1.2–2.9 cm long, 0.20–0.25 cm wide; dorsal sepal 0.7–1.2 cm long, 0.32–0.55 cm wide, oblong-obovate, apex acute, margin entire; lateral sepals 0.7–1.4 cm long, 0.39–0.60 cm wide, obovate, falcate, apex acute, margin entire; petals 0.8–1.3 cm long, 0.30–0.59 cm wide, obovate to elliptic, apex acute, margin entire thoughout or apical half erose. Lip 0.30–0.67 cm long, 0.9–1.6 cm wide, united with column, 3-lobed, base of the disk with a pair of ovoid callus, and a longitudinal keel 0.25–0.48 cm long, 0.14–0.30 cm wide; lateral lobes 0.35–0.70 cm long, 0.40–0.75 cm wide, semiorbicular, margin serrate to fimbriate, midlobe 0.22–0.38 cm long, 0.30–0.68 cm wide, flabellate, apex bilobed, margin serrate to fimbriate. Column 0.5–1.1 cm long, 0.30–0.35 cm wide, apex of ventral face with a pair of projections on both sides, directed to lip base. Anther apical, ovate. Pollinia 4, ovoid, laterally compressed. Cuniculus 0.7–0.9 cm long, penetrating about half the ovary. Capsule 5.0–5.2 cm long, 1.3–1.9 cm wide, globose to ovoid. Figure 5.

Paratypes. – BRAZIL. Alagoas, Quebrangulo, REBIO Pedra Talhada, 09°15′17″ S 36°25′36″ W, 25 Out 2011, *B. Amorim 1149* (UFP); Paraíba, São João do Tigre, APA das Onças, 6 Jul 2005, *Dantas & al. 1442* (JPB); Pernambuco, Bonito, Reserva municipal, 20 Jul 1995, *M. Alves & al. 34545* (UFP!); ibid. Brejo da Madre de Deus, Fazenda Bituri, 19 Apr 1959, *D. Andrade-Lima 59-3354* (IPA); ibid. São Caetano, RPPN Pedra do Cachorro, 08°14′06″ S 36°11′25.5″ W, 14 Jul 2007, *P. Gomes 684* (UFP!, MO!); ibid. São Caetano, RPPN Pedra do Cachorro, 08°14′ 21.8″ S 36°11′20.3″ W, 29 Aug 2010, *D. Cavalcanti & al. 289* (UFP!, PEUFR!).

Etymology. – The new species is named on behalf of the color of its flowers, which are mainly red, orange and yellow, similar to a flame.

Distribution and ecology – *Epidendrum flammeus* is known from the states of Pernambuco, Paraíba and Alagoas in the northern part of northeastern Brazil (Fig. 1). The species occurs in granitic rock outcrops (up to 1000 m high) on the Borborema plateau, within the Caatinga biome. It grows in association with other rupicolous plants such as bromeliads and orchid in islands of vegetation. Flowers have been observed during the entire year, with a clear flowering peak from December to March.

Many inventories from northeastern Brazil (Pereira, 1981; Gomes-Ferreira, 1990; Felix & Carvalho, 2002) list specimens of *E. flammeus* under the name *E. fulgens*. However, *E. flammeus* is limited to the north of northeastern Brazil (Borborema plateau) where it grows exclusively on granitic rock outcrops.

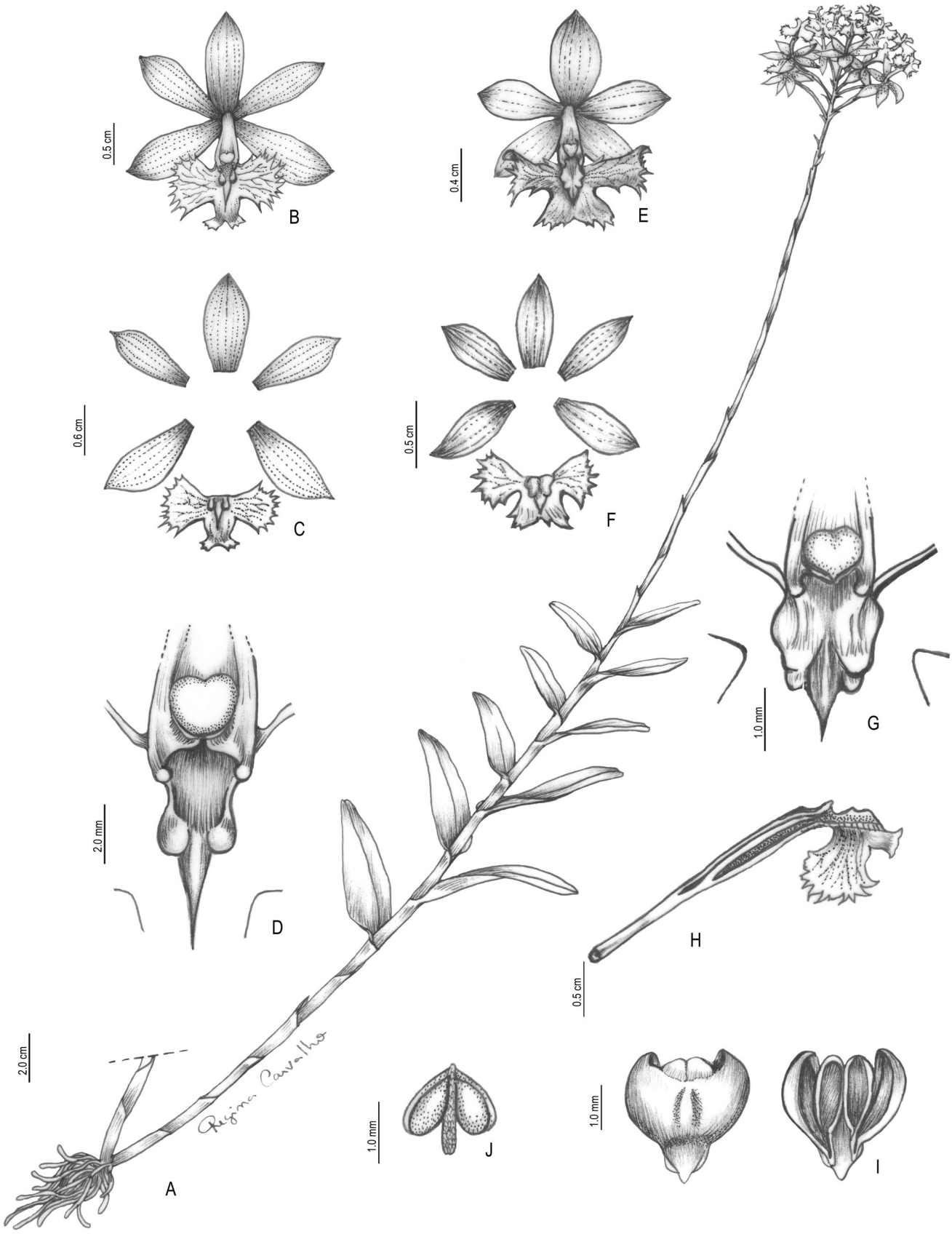

Fig. 5. *Epidendrum flammeus*: **A,** habit; **B–C,** yellow flowers; **D,** yellow flower callus; **E–F,** red flowers; **G,** red flower callus; **H,** longitudinal section of pedicellate ovary; **I,** anther; **J,** pollinarium.

Epidendrum fulgens and *E. puniceoluteum* grow in southern and southeastern Brazil, in coastal sand areas locally called "restingas" (Barros & al., 2011). *Epidendrum cinnabarinum* is known from "restingas" and the granitic rock outcrops found in the Caatinga ecosystem in northeastern Brazil, and *E. denticulatum* occurs in southern, southeastern, and southern parts of northeastern Brazil, also growing on sandy soils along the coast and in savanna vegetation (Cerrado) (Barros & al., 2011, Fig. 1).

Morphological affinities. – The new species described here has previously been wrongly identified as *E. fulgens*, because of similarities in flower color which ranges from red to orange or yellow in the latter species (Fig. S1). The morphology of the sepals, petals and lip are also similar in these two species, as well as in all species of *Epidendrum* placed in the Atlantic clade.

Epidendrum fulgens and *E. cinnabarinum* share a similar column morphology, but both species lack the pair of lateral projections on the ventral face of the apex directed to the lip's base seen in other species of the Atlantic clade (*E. denticulatum*, *E. flammeus*, *E. punniceoluteum*). Although *E. flammeus* and *E. cinnabarinum* are sympatric (Fig. 1), their flowers differ in size and morphology. In *E. puniceoluteum*, the flowers are red-purple and larger than in *E. denticulatum* and *E. flammeus*, which have flowers of similar size. *Epidendrum denticulatum* has pink flowers, whereas in *E. flammeus* they are yellow, orange or red. In addition, *E. flammeus* shows a curved lip, which is erect in *E. denticulatum*.

Two morphotypes can be recognized in the populations of *E. flammeus* (Fig. 5). In one of them, plants have yellow and slightly larger flowers, and the lip is more incurved. The second morphotype has red and smaller flowers and the lip is only slightly incurved. Specimens with intermediate characteristics such as orange flowers, and with variation in flower size and lip curvature are common within populations. Clear discontinuities between morphotypes were not observed in the morphometric analysis (Fig. 2). Fine-scale genetic studies within populations could clarify the role of microhabitat preferences between morphotypes, and a pollination survey could clarify the role of color selection and the maintenance of the flower variability in this species.

Key to *Epidendrum flammeus* and related species of the Atlantic clade of subg. *Amphyglottium*

1. Dorsal sepal >4 cm long; lateral lobes of the lip fimbriate ..... ***E. cinnabarinum***
1. Dorsal sepal <4cm long; lateral lobes of the lip denticulate to erose 2
2. Pink flowers ..... ***E. denticulatum***
2. Flowers orange, purpureous, red or yellow 3
3. Pair of lateral projections of column absent ... ***E. fulgens***
3. Pair of lateral projections of ventral face of column apex directed to lip base present 4
4. Lip ≥15 mm wide; cuniculous ≥ 1/2 as long as pedicelate ovary ..... ***E. puniceoluteum***
4. Lip <15 mm wide; cuniculous <1/2 as long as pedicelate ovary ..... ***E. flammeus***

■ ACKNOWLEDGEMENTS

We thank Kalinne Mendes and Guaraci Cardoso for help during field work, Rafael B. Louzada for assistance with ML analysis, and Salvatore Cozzolino for valuable comments. Funding for this study was provided by fellowship from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP-2009/15052-0) to FP, from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) to EP and AA, and grants from FAPESP (2009/52725-3; 2009/174118) to CP and from NSF, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Benefia Foundation to MA. The authors thank Regina Carvalho for the line drawing of the new species.

■ LITERATURE CITED

Almeida, A.M. & Figueiredo, R.A. 2003. Ants visit nectaries of *Epidendrum denticulatum* (Orchidaceae) in a Brazilian rainforest: Effects on herbivory and pollination. *Brazil. J. Biol.* 63: 551–558.

Antonelli, A. & Sanmartín, I. 2011. Why are there so many plant species in the Neotropics? *Taxon* 60: 403–414.

Barbará, T., Palma-Silva, C., Paggi, G.M., Bered, F., Fay, M.F. & Lexer, C. 2007. Cross-species transfer of nuclear microsatellite markers: Potential and limitations. *Molec. Ecol.* 16: 3759–3767.

Barrett, C.F. & Freudenstein, J.V. 2011. An integrative approach to delimiting species in a rare but widespread mycoheterotrophic orchid. *Molec. Ecol.* 20: 2771–2786.

Barros, F. de, Vinhos, F., Rodrigues, V.T., Fraga, C.N. & Pessoa, E.M. 2011. Orchidaceae. In: Lista de Espécies da Flora do Brasil. Jardim Botânico do Rio de Janeiro. http://floradobrasil.jbrj.gov.br/2011/FB000179, accessed 20 Apr. 2012).

Bierzychudek, P. 1981. *Asclepias, Lantana* and *Epidendrum*: A floral mimicry complex? *Biotropica* 13: 54–58.

Borba, E.L., Shepherd, G.J., Van den Berg, C. & Semir, J. 2002. Floral and vegetative morphometrics of five *Pleurothallis* (Orchidaceae) species: Correlations with taxonomy, phylogeny, genetic variability, and pollination systems. *Ann. Bot.* 90: 219–230

Buzzato, C.R., Davies, K.L., Singer, R.B., Santos, R.P. & Van den Berg, C. 2012. A comparative survey of floral characters in *Capanemia* (Orchidaceae: Oncidiinae). *Ann. Bot.* 109: 135–144.

Cozzolino, S. & Widmer, A. 2005. Orchid diversity: An evolutionary consequence of deception? *Trends Ecol. Evol.* 20: 487–494.

Cracraft, J. 1983. Species concept and speciation analysis. *Curr. Ornithol.* 1: 159–187.

Davis, J.I. & Nixon, K.C. 1992. Populations, genetic variation, and the delimitation of phylogenetic species. *Syst. Biol.* 41: 421–435.

Dayrat, B. 2005. Towards integrative taxonomy. *Biol. J. Linn. Soc.* 85: 407–415.

De Queiroz, K. 1998. The general lineage concept of species, species criteria, and the process of speciation. Pp. 57–75 in: Howard, D.J. & Berlocher, S.H. (eds.), *Endless forms: Species and speciation*. Oxford: Oxford University Press.

De Queiroz, K. & Donoghue, M. 1988. Phylogenetic systematics and the species problem. *Cladistics* 4: 317–338.

Diniz-Filho, J.A.F., Telles, M.P.C., Bonatto, S.L., Eizirik, E., Freitas, T.R.O., Marco, P., Santos, F.R., Sole-Cava, A. & Soares, T.N. 2008. Mapping the evolutionary twilight zone: Molecular markers, populations and geography. *J. Biogeogr.* 35: 753–763.

Du, F., Petit, R. & Liu, J. 2009. More introgression with less gene flow: Chloroplast versus mitochondrial DNA in the *Picea asperata* complex in China, and comparison with other Conifers. *Molec. Ecol.* 18: 1396–1407.

Duminil, J. & Di Michele, M. 2009. Plant species delimitation: A

comparison of morphological and molecular markers. *Pl. Biosyst.* 143: 528–542.

Duminil, J., Caron, H., Scotti, I., Cazal, S.O. & Petit, R.J. 2006. Blind population genetics survey of tropical rainforest trees. *Molec. Ecol.* 15: 3505–3513.

Duminil, J., Fineschi, S., Hampe, A., Jordano, P., Salvini, D., Vendramin, G.G. & Petit, R.J. 2007. Can population genetic structure be predicted from life-history traits? *Amer. Naturalist* 169: 662–672.

Earl, D.A. & Holdt, B.M. von 2011. STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genet. Resources* online, doi: 10.1007/s12686-011-9548.

Evanno, G., Regnaut, S. & Goudet, J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Molec. Ecol.* 14: 2611–2620.

Felix, L.P. & Carvalho, R. 2002. Diversidade de orquídeas no Estado de Pernambuco. Pp. 207–217 in: Tabarelli, M. & Silva, J.M.C. (eds.), *Diagnóstico da biodiversidade de Pernambuco*, vol. 1. Recife: Editora Massangana.

Felsenstein, J. 1985. Confidence-limits on phylogenies with a molecular clock. *Syst. Zool.* 34: 152–161.

Fitch, W.M. 1971. Toward defining the course of evolution: Minimum change for a specific tree topology. *Syst. Zool.* 20: 406–416.

Fuhro, D., Araújo, A.M. & Irgang, B.E. 2010. Are there evidences of a complex mimicry system among *Asclepias curassavica* (Apocynaceae), *Epidendrum fulgens* (Orchidaceae), and *Lantana camara* (Verbenaceae) in southern Brazil? *Revista Brasil. Bot.* 33: 589–598.

Gomes-Ferreira, A.B. 1990. Levantamento das orquídeas do nordeste oriental do Brasil. Pp. 13–38 in: Valle, L.F. (ed.), *II encontro nacional de orquidófilos e orquidólogos*. Rio de Janeiro: Expressão e Cultura.

Hágsater, E. & Soto-Arenas, M.A. 2005. *Epidendrum* L. Pp. 236–251 in: Pridgeon, A.M., Cribb, P.J., Chase, M.W. & Rasmussen, F.N. (eds.), *Genera Orchidacearum*, vol. 4. Oxford: Oxford University Press.

Hall, T.A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids Symp. Ser.* 41: 95–98.

Hausdorf, B. 2011. Progress toward a general species concept. *Evolution* 65: 923–931.

Hausdorf, B. & Hennig, C. 2010. Species delimitation using dominant and codominant multilocus markers. *Syst. Biol.* 59: 491–503.

Hendry, A.P., Lohmann, L.G., Conti, E., Cracraft, J., Crandall, K.A., Faith, D.P., Häuser, C., Joly, C.A., Kogure, K., Larigauderie, A., Magallón, S., Moritz, C., Tillier, S., Zardoya, R., Prieur-Richard, A., Walther, B.A., Yahara, T. & Donoghue, M.J. 2010. Evolutionary biology in biodiversity science, conservation, and policy: A call to action. *Evolution* 64: 1517–1528.

Hollingsworth, P.M., Forrest, L.L., Spouge, J.L., Hajibabaei, M., Ratnasingham, S., Bank, M., Chase, M.W., Cowan, R.S., Erickson, D.L., Fazekas, A., Graham, S., James, K., Kim, K.J., Kress, W., Schneider, H., Van-Alphen-Stahl, J., Barrett, S., Van-den-Berg, C., Bogarin, D., Burgess, K., Cameron, K., Carine, M., Chacón, J., Clark, A., Conrad, F., Devey, D., Clarkson, J., Ford, C., Hedderson, T., Hollingsworth, M., Husband, B., Kelly, L., Kesanakurti, P., Kim, J., Kim, Y., Lahaye, R., Lee, H.L., Long, D., Madriñán, S., Maurin, O., Meusnier, I., Newmaster, S., Park, C.W., Percy, D., Petersen, G., Richardson, J., Salazar, G., Savolainen, V., Seberg, O., Wilkinson, M., Yi, D.K. & Little, D. 2009. A DNA barcode for land plants. *Proc. Natl. Acad. Sci. U.S.A.* 106: 12794–12797.

Hubisz, M.J., Falush, D., Stephens, M. & Pritchard, J.K. 2009. Inferring weak population structure with the assistance of sample group information. *Molec. Ecol. Resources* 9: 1322–1332.

Huelsenbeck, J.P. & Andolfatto, P. 2007. Inference of population structure under a Dirichlet process model. *Genetics* 175: 1787–1802.

Juillet, N. & Scopece, G. 2010. Does floral trait variability enhance reproductive success in deceptive orchids? *Perspect. Pl. Ecol. Evol. Syst.* 12: 317–322.

Knowles, L.L. & Carstens, B.C. 2007. Delimiting species without monophyletic gene trees. *Syst. Biol.* 56: 887–895.

Lahaye, R., Van der Bank, M., Bogarin, D., Warner, J., Pupulin, F., Gigot, G., Maurin, O., Duthoit, S., Barraclough, T.G. & Savolainen, V. 2008. DNA barcoding the floras of biodiversity hotspots. *Proc. Natl. Acad. Sci. U.S.A.* 105: 2923–2928.

Lexer, C., Joseph, J., Van Loo, M., Prenner, G., Heinze, B., Chase, M.W. & Kirkup, D. 2009. The use of digital image-based morphometrics to study the phenotypic mosaic in taxa with porous genomes. *Taxon* 58: 349–364.

Lynch, M. & Walsh, B. 1998. *Genetics and analysis of quantitative traits*. Sunderland: Sinauer.

Mallet, J. 1995. A species definition for the modern synthesis. *Trends Ecol. Evol.* 10: 294–299.

Mayr, E. 1942. *Systematics and the origin of species*. New York: Columbia University Press.

Moccia, M.D., Widmer, A. & Cozzolino, S. 2007. The strength of reproductive isolation in two hybridizing food-deceptive orchid species. *Molec. Ecol.* 16: 2855–66.

Padial, J.M., Miralles, A., De la Riva, I. & Vences, M. 2010. The integrative future of taxonomy. *Frontiers Zool.* 7: 16–30.

Palma-Silva, C., Wendt, T., Pinheiro, F., Barbará, T., Fay, M.F., Cozzolino, S. & Lexer, C. 2011. Sympatric bromeliad species (*Pitcairnia* spp.) facilitate tests of mechanisms involved in species cohesion and reproductive isolation in Neotropical inselbergs. *Molec. Ecol.* 20: 3185–3201.

Pansarin, E.R. & Amaral, M.C.E. 2008. Reproductive biology and pollination mechanisms of *Epidendrum secundum* (Orchidaceae) floral variation: A consequence of natural hybridization? *Pl. Biol.* 10: 211–219.

Pellegrino, G., Musacchio, A., Noce, M.E., Palermo, A.M. & Widmer, A. 2005. Reproductive versus floral isolation among morphologically similar *Serapias* L. species (Orchidaceae). *J. Heredity* 96: 15–23.

Pereira, L.A. 1981. *Orquídeas de Alagoas*. Maceió: Sergasa.

Petit, R.J. & Excoffier, L. 2009. Gene flow and species delimitation. *Trends Ecol. Evol.* 24: 386–393.

Petit, R.J., Duminil, J., Fineschi, S., Hampe, A., Salvini, D. & Vendramin, G.G. 2005. Comparative organization of chloroplast, mitochondrial and nuclear diversity in plant populations. *Molec. Ecol.* 14: 689–701.

Pillon, Y., Munzinger, J., Amir, H., Hopkins, H.C.F. & Chase, M.W. 2009. Reticulate evolution on a mosaic of soils: Diversification of the New Caledonian endemic genus *Codia* (Cunoniaceae). *Molec. Ecol.* 18: 2263–2275.

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

Pinheiro, F. & Barros, F. 2007a. Morphometric analysis of *Epidendrum secundum* (Orchidaceae) in southeastern Brazil. *Nord. J. Bot.* 25: 129–136.

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

Pinheiro, F., Barros, F., Palma-Silva, C., Fay, M.F., Lexer, C. & Cozzolino, S. 2011. Phylogeography and genetic differentiation along the distributional range of the orchid *Epidendrum fulgens*: A Neotropical coastal species not restricted to glacial refugia. *J. Biogeogr.* 38: 1923–1935.

Pinheiro, F., Barros, F.D., Palma-Silva, C., Meyer, D., Fay, M.F., Suzuki, R.M., Lexer, C. & Cozzolino, S. 2010. Hybridization and introgression across different ploidy levels in the Neotropical orchids *Epidendrum fulgens* and *E. puniceoluteum* (Orchidaceae). *Molec. Ecol.* 19: 3981–3994.

Pinheiro, F., Koehler, S., Corrêa, A.M., Salatino, M.L.F., Salatino, A.

& Barros, F. 2009a. Phylogenetic relationships and infrageneric classification of *Epidendrum* subgenus *Amphiglottium* (Laeliinae, Orchidaceae). *Pl. Syst. Evol*. 283: 165–177.

Pinheiro, F., Palma-Silva, C., Barros, F. & Cozzolino, S. 2009b. Cross-amplification and characterization of microsatellite loci for the Neotropical orchid genus *Epidendrum*. *Genet. Molec. Biol*. 32: 337–339.

Pinheiro, F., Palma-Silva, C., Barros, F., Félix, L.P., Lexer, C., Cozzolino, S. & Fay, M.F. 2009c. Chloroplast microsatellite markers for the Neotropical orchid genus *Epidendrum*, and cross-amplification in other Laeliinae species (Orchidaceae). *Conservation Genet. Resources* 1: 505–511.

Pinheiro, F., Santos, M.O., Barros, F., Meyer, D., Salatino, A., Souza, A.P. & Cozzolino, S. 2008a. Isolation and characterization of microsatellite loci in the Brazilian orchid *Epidendrum fulgens*. *Conservation Genet*. 9: 1661–1663.

Pinheiro, F., Santos, M.O., Palma-Silva, C., Barros, F., Meyer, D., Salatino, A., Souza, A.P. & Cozzolino, S. 2008b. Isolation and characterization of microsatellite loci in *Epidendrum puniceoluteum*, an endemic orchid from the Atlantic Rainforest. *Molec. Ecol. Resources* 8: 1114–1116.

Pires, A.C. & Marinoni, L. 2010. DNA barcoding and traditional taxonomy unified through Integrative Taxonomy: A view that challenges the debate questioning both methodologies. *Biota Neotrop.* 10: 339–346.

Posada, D. 2008. jModelTest: Phylogenetic model averaging. *Molec. Biol. Evol*. 25: 1253–1256.

Pritchard, J.K., Wen, X. & Falush, D. 2010. Documentation for STRUCTURE software, version 2.3. http://pritch.bsd.uchicago.edu/structure.html.

Reeves, P.A. & Richards, C.M. 2011. Species delimitation under the general lineage concept: An empirical example using wild North American hops (Cannabaceae: *Humulus lupulus*). *Syst. Biol*. 60: 45–59.

Savolainen, V., Anstett, M.C., Lexer, C., Hutton, I., Clarkson, J.J., Norup, M.V., Powell, M.P., Springate, D., Salamin, N. & Baker. W.J. 2006. Sympatric speciation in palms on an oceanic island. *Nature* 441: 210–213.

Schlick-Steiner, B.C., Steiner, F.M., Seifert, B., Stauffer, C., Christian, E. & Crozier, R.H. 2010. Integrative taxonomy: A multisource approach to exploring biodiversity. *Annual Rev. Entomol.* 55: 421–438.

Shaw, J., Lickey, E.B., Beck, J.T., Farmer, S.B., Liu, W., Miller, J., Siripun, K.C., Winder, C.T., Schilling, E.E. & Small, R.L. 2005. The tortoise and the hare II: Relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. *Amer. J. Bot.* 92: 142–166.

Shaw, J., Lickey, E.B., Schilling, E.E. & Small, R.L. 2007. Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: The tortoise and the hare III. *Amer. J. Bot.* 94: 275–288.

Silvestro, D. & Michalak, I. 2011. raxmlGUI: A graphical front-end for RAxML. *Organisms Diversity Evol*. online, doi: 10.1007/s13127-011-0056-0.

Sites, J.W. & Marshall, J.C. 2004. Operational criteria for delimiting species. *Annual Rev. Ecol. Evol. Syst.* 35: 199–227.

Sokal, R.R. & Crovello, T.J. 1970. The biological species concept: A critical evaluation. *Amer. Naturalist* 104: 127–153.

Stamatakis, A. 2006. RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22: 2688–2690.

Swofford, D.L. 2002. PAUP*: Phylogenetic analysis using parsimony (*and other methods), version 4. Sunderland, Massachusetts: Sinauer.

Waser, N.M. 1998. Pollination, angiosperm speciation, and the nature of species boundaries. *Oikos* 81: 198–201.

Wu, C.I. 2001. The genic view of the process of speciation. *J. Evol. Biol*. 14: 851–865.

Appendix. Specimens used in morphological and molecular analyses with voucher information, country, locality, and GenBank accession numbers for *rpl32-trnL*, *trnT-trnL* and *trnL-trnF*, respectively. Newly generated sequences are indicated by an asterisk.

Epidendrum cinnabarinum Salzm. ex Lindl., Brazil, Sergipe, Pirambu, *F. Pinheiro 609* (SP), JQ645971*, JQ645991*, JQ646011*; ***Epidendrum cinnabarinum***, Brazil, Bahia, Lagoa do Abaeté, *F. Pinheiro 608* (SP), JQ645970*, JQ645990*, JQ646010*; ***Epidendrum denticulatum*** Barb. Rodr., Brazil, Bahia, Olivença, *F. Pinheiro 626* (SP); ***Epidendrum denticulatum***, Brazil, Bahia, Alcobaça, *F. Pinheiro 627* (SP); ***Epidendrum denticulatum***, Brazil, Minas Gerais, Poços de Caldas, *F. Pinheiro 628* (SP); ***Epidendrum denticulatum***, Brazil, Rio de Janeiro, Marambaia, *F. Pinheiro 610* (SP), JQ645972*, JQ645992*, JQ646012*; ***Epidendrum denticulatum***, Brazil, São Paulo, Botucatu, *F. Pinheiro 629* (SP); ***Epidendrum denticulatum***, Brazil, São Paulo, São Paulo, *F. Pinheiro 630* (SP); ***Epidendrum denticulatum***, Brazil, São Paulo, Itapeva, *F. Pinheiro 611* (SP), JQ645973*, JQ645993*, JQ646013*; ***Epidendrum flammeus*** E. Pessoa & M. Alves, Brazil, Pernambuco, Pedra do Cachorro, *F. Pinheiro 612*, JQ645974*, JQ645994*, JQ646014*; ***Epidendrum flammeus***, Brazil, Pernambuco, Pedra do Cachorro, *F. Pinheiro 613*, JQ645975*, JQ645995*, JQ646015*; ***Epidendrum flammeus***, Brazil, Pernambuco, Pedra do Cachorro, *F. Pinheiro 614*, JQ645976*, JQ645996*, JQ646016*; ***Epidendrum flammeus***, Brazil, Pernambuco, Pedra do Cachorro, *F. Pinheiro 615*, JQ645977*, JQ645997*, JQ646017*; ***Epidendrum fulgens*** Brongn., Brazil, Rio de Janeiro, Parati, *F. Pinheiro 631* (SP), JQ645978*, JQ645998*, JQ646018*; ***Epidendrum fulgens***, Brazil, São Paulo, Bertioga, *F. Pinheiro 616* (SP); ***Epidendrum fulgens***, Brazil, São Paulo, Cananéia, *F. Pinheiro 632* (SP); ***Epidendrum fulgens***, Brazil, Santa Catarina, Itajaí, *F. Pinheiro 633* (SP); ***Epidendrum fulgens***, Brazil, Santa Catarina, Florianópolis, *F. Pinheiro 634* (SP); ***Epidendrum fulgens***, Brazil, Santa Catarina, Imbituba, *F. Pinheiro 617* (SP), JQ645979*, JQ645999*, JQ646019*; ***Epidendrum fulgens***, Brazil, Rio Grande do Sul, Porto Alegre, *F. Pinheiro 635* (SP); ***Epidendrum puniceoluteum*** Pinheiro & Barros, Brazil, São Paulo, Ilha Comprida, *F. Pinheiro 622* (SP), JQ645984*, JQ646004*, JQ646024*; ***Epidendrum puniceoluteum***, Brazil, São Paulo, Cananéia, *F. Pinheiro 636* (SP); ***Epidendrum puniceoluteum***, Brazil, Paraná, Pontal do Sul, *F. Pinheiro 621* (SP), JQ645983*, JQ646003*, JQ646023*; ***Epidendrum calanthum*** Rchb.f. & Warsz., Brazil, Roraima, Serra Pacaraima, *F. Pinheiro 606* (SP), JQ645968*, JQ645988*, JQ646008*; ***Epidendrum macrocarpum*** Rich., Brazil, Pernambuco, Recife, *F. Pinheiro 618* (SP), JQ645980*, JQ646000*, JQ646020*; ***Epidendrum myrmecophorum*** Barb. Rodr., Brazil, Rio de Janeiro,Araruama, *F. Pinheiro 619* (SP), JQ645981*, JQ646001*, JQ646021*; ***Epidendrum radicans*** Pav. ex Lindl., Mexico, Oaxaca, *F. Pinheiro 623* (SP), JQ645985*, JQ646005*, JQ646025*; ***Epidendrum secundum*** Jacq., Brazil, Santa Catarina, Serra do Rio do Rastro, *F. Pinheiro 624* (SP), JQ645986*, JQ646006*, JQ646026*; ***Epidendrum xanthinum*** Lindl., Brazil, Minas Gerais, Santa Bárbara, *F. Pinheiro 625* (SP), JQ645987*, JQ646007*, JQ646027*. **OUTGROUP:** ***Epidendrum campestre*** Lindl., Brazil, Minas Gerais, Santana do Riacho, *F. Pinheiro 607* (SP), JQ645969*, JQ645989*, JQ646009*; ***Epidendrum nocturnum*** Jacq., Brazil, São Paulo, Cananéia, *F. Pinheiro 620* (SP), JQ645982*, JQ646002*, JQ646022*.

Vol. 61 • August 2012

TAXON

International Journal of Taxonomy, Phylogeny and Evolution

Electronic Supplement to

Integrating different tools to disentangle species complexes: A case study in *Epidendrum* (Orchidaceae)

Edlley Max Pessoa, Marccus Alves, Anderson Alves-Araújo, Clarisse Palma-Silva & Fábio Pinheiro

Taxon 61: 721–734

Table S1. Morphological characters used in morphometric analyses, means and standard deviations of *Epidendrum cinnabarinum* (Ec), *E. denticulatum* (Ed), *E. flammeus* (Efl), *E. fulgens* (Ef) and *E. puniceoluteum* (Ep) and results of discriminant analysis, including F-to-remove and canonical discriminant values for the first three axes (CA 1, CA 2 and CA 3).

Characters	Ec	Ed	Efl	Ef	Ep	F-to-remove	CA 1	CA 2	CA 3
Pedicel length	31.56 ± 4.80	21.75 ± 4.24	19.27 ± 3.60	19.56 ± 2.92	25.50 ± 3.34	15.12	0.070	0.099	0.052
Dorsal sepal length	20.15 ± 1.45	10.54 ± 1.08	10.18 ± 1.36	13.39 ± 0.98	14.48 ± 1.25	0.74	−0.015	0.107	−0.183
Dorsal sepal width	5.94 ± 0.91	3.85 ± 0.44	4.26 ± 0.52	5.34 ± 0.50	5.72 ± 0.53	3.06	−0.062	−0.283	0.443
Lateral sepal length	21.56 ± 2.28	11.11 ± 1.07	10.53 ± 1.43	14.06 ± 1.04	15.13 ± 1.29	2.73	−0.004	0.199	0.067
Lateral sepal width	5.72 ± 0.62	4.13 ± 0.52	4.70 ± 0.57	5.80 ± 0.46	6.24 ± 0.65	15.21	−0.459	−0.897	−1.032
Petal length	21.24 ± 2.43	10.95 ± 1.02	10.29 ± 1.29	13.31 ± 0.91	14.82 ± 1.28	7.07	−0.194	0.403	−0.269
Petal width	5.23 ± 1.02	3.63 ± 0.64	4.27 ± 0.72	5.44 ± 0.81	5.82 ± 0.75	4.19	0.082	−0.13	0.325
Lip length	8.29 ± 1.16	6.59 ± 1.00	5.25 ± 0.77	6.05 ± 0.59	6.58 ± 0.65	20.96	0.315	0.208	0.617
Lip width	15.41 ± 1.79	12.14 ± 1.62	12.09 ± 1.58	14.56 ± 1.38	17.09 ± 1.51	18.72	−0.226	0.013	−0.443
Column length	14.56 ± 1.11	6.82 ± 0.91	7.98 ± 1.36	12.21 ± 0.89	10.38 ± 0.87	58.59	−0.493	−1.000	−0.013
Lateral lobe of lip length	7.06 ± 0.36	6.31 ± 0.88	5.15 ± 0.83	6.25 ± 0.67	7.33 ± 0.67	19.26	0.838	0.423	0.279
Lateral lobe of lip width	7.42 ± 0.65	6.05 ± 0.98	5.77 ± 0.86	8.27 ± 0.98	7.80 ± 1.16	20.09	−0.241	−0.276	0.359
Central lobe of lip length	5.54 ± 0.50	4.28 ± 0.86	3.11 ± 0.39	3.38 ± 0.49	5.15 ± 0.61	21.6	0.201	0.679	−0.629
Central lobe of lip width	3.70 ± 0.34	6.46 ± 1.00	4.85 ± 0.92	4.69 ± 0.78	7.20 ± 1.22	23.82	0.482	−0.093	−0.300
Callus of lip length	7.81 ± 0.35	2.71 ± 0.41	3.51 ± 0.54	4.43 ± 0.51	4.04 ± 0.58	77.87	−1.404	0.441	0.453
Callus of lip width	1.58 ± 0.19	2.00 ± 0.40	2.35 ± 0.44	2.48 ± 0.39	3.14 ± 0.56	15.46	0.545	−0.596	−1.001

Table S2. Results of jackknifed classification matrix from discriminant analysis performed on 420 individuals from *Epidendrum cinnabarinum* (38), *E. denticulatum* (79), *E. flammeus* (51), *E. fulgens* (186) and *E. puniceoluteum* (66), indicating the percentage of correct classification of individuals in each species. Wilks' Lambda = 0.011, $P = 0.0000$.

Species	*E. cinnabarinum*	*E. denticulatum*	*E. flammeus*	*E. fulgens*	*E. puniceoluteum*	% correct
E. cinnabarinum	38	0	0	0	0	100
E. denticulatum	0	76	3	0	0	96
E. flammeus	0	0	48	3	0	94
E. fulgens	0	0	0	186	0	100
E. puniceoluteum	0	1	0	0	65	98
Total	38	77	51	189	65	98

Fig. S1. Morphological and color variation within and among *Epidendrum* species from the Atlantic clade.

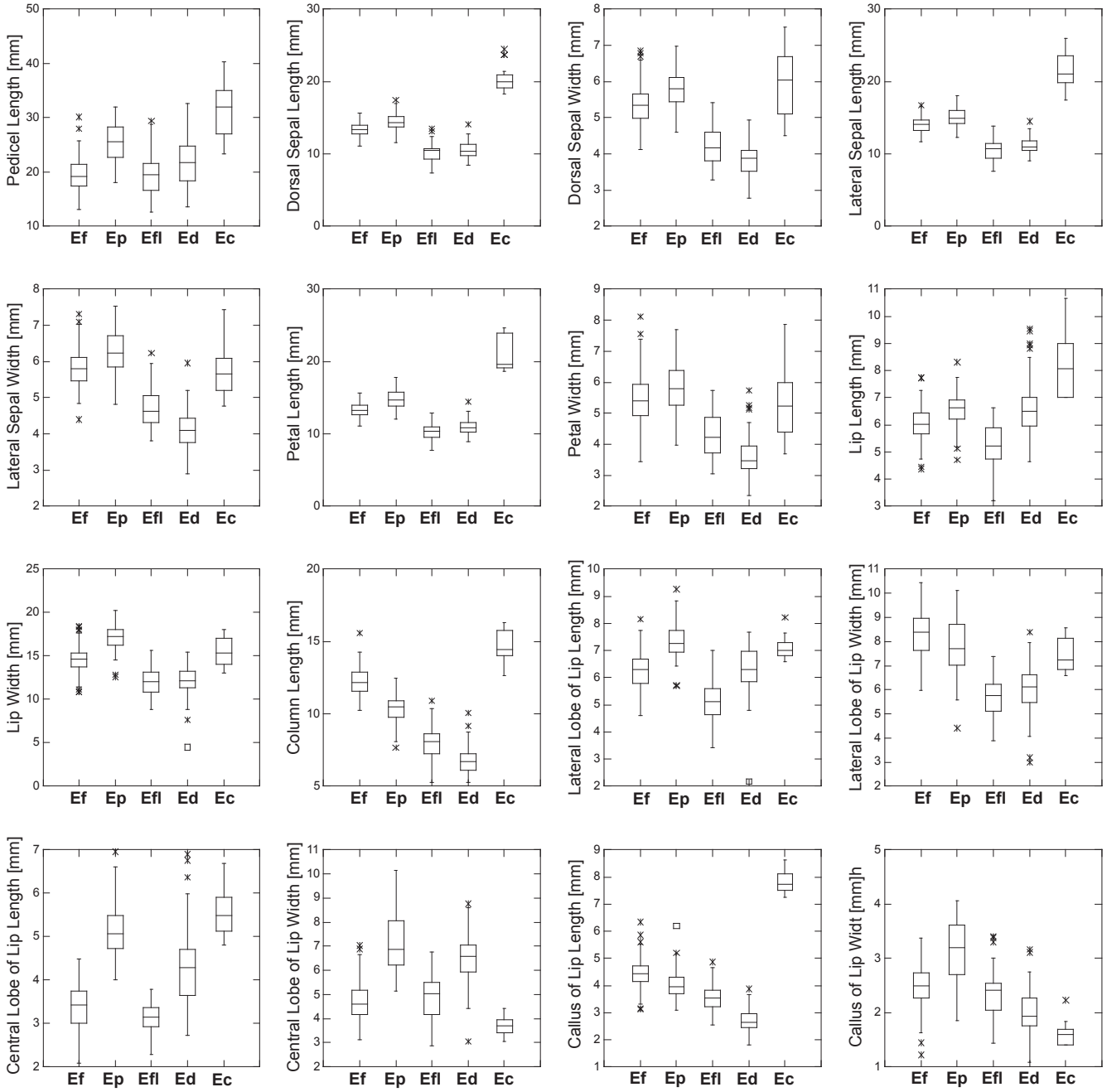

Fig. S2. Box-plots for the 16 quantitative characters measured on *Epidendrum fulgens* (Ef), *E. puniceoluteum* (Ep), *E. flammeus* (Efl), *E. denticulatum* (Ed) and *E. cinnabarinum* (Ec.). Rectangles define 25 and 75 percentiles; horizontal lines show median; whiskers are from 10 to 90 percentile; asterisks indicate extreme values.

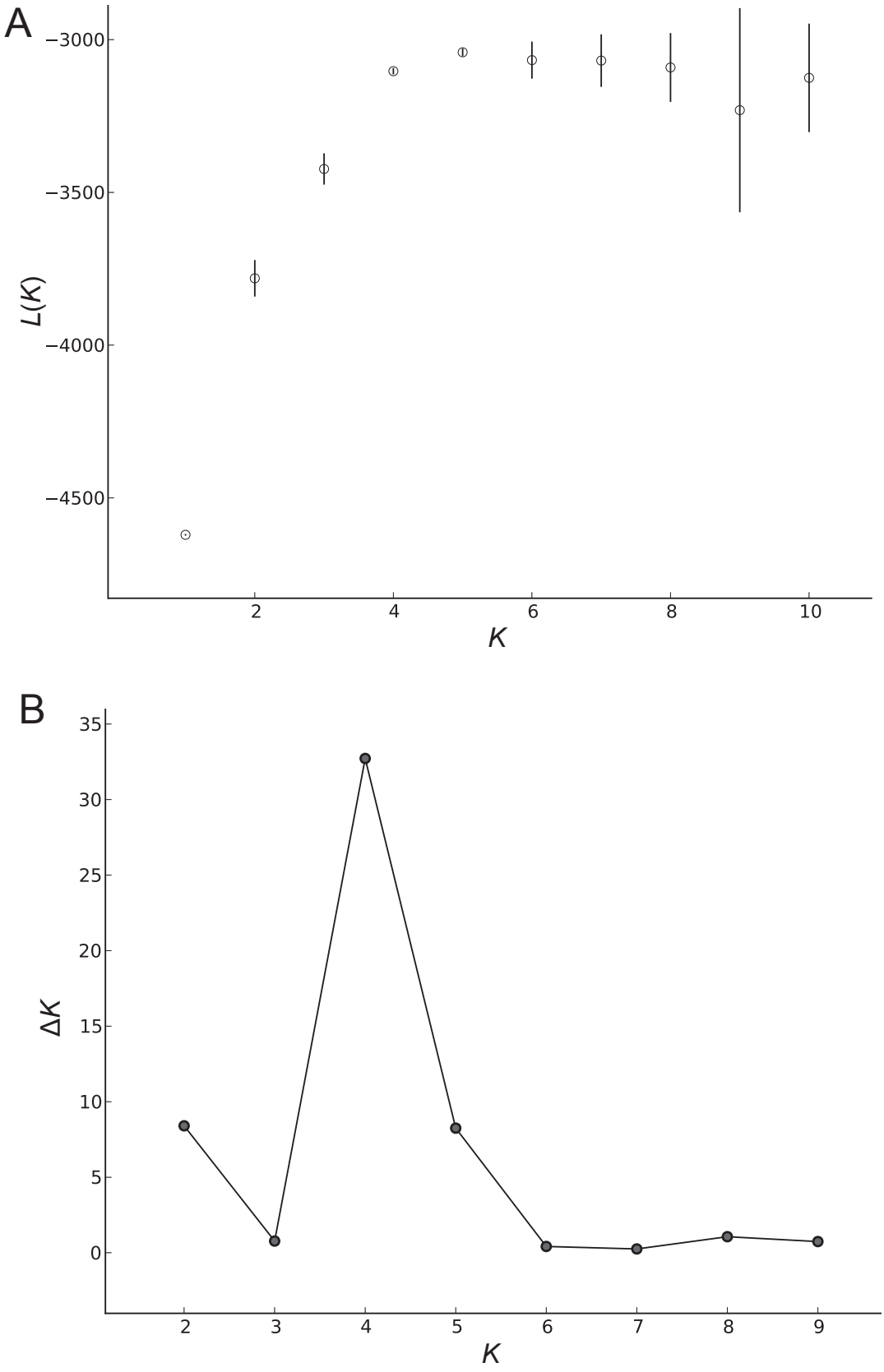

Fig. S3. Graphical methods allowing detection of the true number of groups *K*, based on ad hoc statistics provided by STRUCTURE. **A,** Mean log probability of data $L(K)$ (±SD) as a function of *K*, where the values plateaus at $K = 4$; **B,** the magnitude of ΔK as a function of *K*, where the modal value of this distribution indicates the true *K* or the uppermost level of structure, here four clusters.