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ORIGINAL ARTICLE

Interspecific genetic analysis of orchids in Brazil using molecular markers

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Abstract Several species of Orchidaceae, one of the largest plant families, are considered endangered throughout South America and legal protection policies are needed so they can be preserved. Inter simple sequence repeats (ISSRs) markers are a potential tool to be used in the phylogenetic reconstruction of closely related species. In this study, we evaluate the polymorphic information content (PIC) and optimum number of ISSR markers (ONM) for five Laeliinae orchids in order to assess genetic diversity. The phylogenetic relationships between *Cattleya granulosa*, an endangered Brazilian orchid, and four other native Brazilian species (*Brassavola tuberculata*, *Cattleya bicolor*, *Cattleya labiata* and *Cattleya schofieldiana*) were analyzed for genetic diversity and differentiation. The 11 selected primers generated 166 unambiguous loci (PIC = 0.354; ONM = 156). Of the five studied species, *C. bicolor* exhibited the highest level of genetic diversity ($H_E = 0.219$), while *C. labiata* exhibited the lowest level ($H_E = 0.132$). The percentage of genetic variation among species (analysis of molecular variance) was 23.26 %. The principal component analysis (PCA) of ISSR data showed that unifoliate and bifoliolate species are genetically divergent. Additionally, PCA indicated a close relation between *C. granulosa* and *C. schofieldiana*, a species considered to be a variety of *C. granulosa* by many researchers. Thus, we conclude that ISSR genetic markers are effective in detecting genetic differentiation among orchid species.

Keywords *Brassavola tuberculata* · *Cattleya* · ISSR · Genetic differentiation · Laeliinae · Orchidaceae

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Introduction

With approximately 24,000 recognized species and about 800 genera, Orchidaceae is one of the largest plant families (World Checklist of the Monocotyledons 2006). The family presents some of the most complex and intriguing pollination mechanisms ever recorded (Lovisa et al. 2010) and its flowers are pollinated by diverse groups of insects and birds (Dodson et al. 1969). Within this family, Laeliinae is a strictly Neotropical subtribe with approximately 1,500 species and around 50 genera; it is the second largest subtribe of Orchidaceae (van den Berg et al. 2009). While artificial hybridization can be employed to combine various genera for horticultural purposes, many interspecific and intergeneric hybrids have also been found in natural habitats (Azevedo et al. 2006; van den Berg et al. 2009; Pinheiro et al. 2010; Storti et al. 2011). Hybridization in nature has been documented through the identification of several intra- and intergeneric species, especially involving *Cattleya*, one of the most economically important genus. Throughout South America, several species of this genus are considered endangered (MMA 2008) and legal policies for their protection are necessary.

Inter simple sequence repeats (ISSRs) have been used extensively to characterize genetic diversity in orchids,

such as *Anoectochilus formosanus* (Zhang et al. 2010), *Balanophora fungosa* (Hsiao et al. 2010), *Cymbidium goeringii* (Wang et al. 2009a), and *Vanilla* species (Verma et al. 2009). Through ISSR markers it is also possible to verify the genetic stability of micropropagated plantlets from tissue culture (e.g., *Vanda stangeana*, Kishor and Devi 2009). ISSR markers can be useful in informing genetic conservation and sustainable use strategies (e.g., *Cymbidium ensifolium*, Wang et al. 2011). However, genetic analyses of endemic South American orchids are still rare (Ávila-Diaz and Oyama 2007; Borba et al. 2001).

The ISSR technique has the ability to generate a large number of multilocus markers and can be applied to analyze almost any organism (Arcade et al. 2000). ISSR is one of many fingerprinting techniques based on polymerase chain reaction (PCR) and is defined as a variant of PCR using sequence primers to amplify simple and repetitive regions between target sequences (Zietjiewicz et al. 1994). The ISSR method assesses the abundance and random distribution of simple sequence repeats (SSRs) in genomes of plants and other organisms, amplifying DNA sequences contained between these SSRs (Reddy et al. 2002). Moreover, ISSR studies at the population level have recently demonstrated the hyper-variable nature of these markers and their potential use in population analyses (Chu-Shu Zhang et al. 2013; Pinheiro et al. 2010).

Inter simple sequence repeats are especially valuable when studying closely related taxa, due to the difficulty in finding DNA regions that are sufficiently variable in recently radiated taxa (Archibald et al. 2006). In a previous study, phylogenetic analysis of the Laeliinae subtribe was performed with ribosomal ITS sequences and plastidal DNA; however, inconsistencies in the trees were reported (van den Berg et al. 2000, 2009). Added to the sequencing data, dominant ISSR markers have been proposed by several authors as being potentially useful for phylogenetic reconstruction of closely related species (Joshi et al. 2000; Hao et al. 2002; Sudupak 2004). Thus, this study assesses the use of a further genetic marker technique in an attempt to address this lack of genetic resolution. Furthermore, we analyze the phylogenetic relationships of the endangered Brazilian orchids *Cattleya granulosa* Lindl., *Cattleya labiata* Lindl. and *Cattleya schofieldiana* Rchb.f. (Martinelli and Moraes 2013; MMA 2008), with two other native Brazilian species of Epidendroideae: *Brassavola tuberculata* Hook. and *Cattleya bicolor* Lindl.

Materials and methods

Plant samples

Fresh leaves were collected from five individuals of each orchid, placed in 2.0-mL tubes containing 10 % CTAB (cetyltrimethyl ammonium bromide), and stored at −20 °C in the laboratory. The species presents a low population density, so the sample size obtained was small. Individuals of *C. granulosa* were sampled from the Atlantic Rainforest in the northeast of Brazil (Municipality of Ceará-Mirim, Rio Grande do Norte State), along with samples of *B. tuberculata* (Municipality of Rio do Fogo, Rio Grande do Norte State), and *C. labiata* (Paraíba State). Specimens of *C. schofieldiana* and *C. bicolor* were donated by private collectors from the Brazilian southeast.

DNA extraction and ISSR amplification

Genomic DNA was extracted from leaves using the CTAB protocol (Doyle and Doyle 1987). Of the 20 screened primers, we selected 11 primers for use in this study (Table 1). PCR amplifications were performed in 12-μL reaction containing DNA 2 ng, PCR buffer (500 mM KCl, 100 mM pH 8.4; 1 % Triton X-100, 20 mM $MgCl_2$), dNTP (2.5 mM), plus an additional 50 mM $MgCl_2$, 1 U *Taq* DNA polymerase, and ISSR primer (2 μM). All amplifications were carried out in a MJ Research PTC-100 thermocycler using the following sequence: an initial step of 2 min at 94 °C; followed by 37 cycles of 15 s at 94 °C, 30 s at 42 °C and 1 min at 72 °C; and a final extension step of 7 min at 72 °C. Amplification products were resolved by electrophoresis on 1.5 % agarose gel, running at 100 V for 3.5 h. Gels were stained with GelRed™ in 1× TAE (Tris–acetate–EDTA) buffer and photographed under ultraviolet light. Molecular weights were estimated using a 1,000-bp DNA ladder. The criterion for choosing primers was that they had unambiguous and reproducible bands.

Identification of the optimal number of bands

The optimum number of ISSR markers (ONM) was calculated using the GENES software (Cruz 2001), through bootstrap analysis (Manly 1997). For each pair of plants, the genetic similarity was estimated from 30 simulations with different sizes (1, 6, 11, …, 161 bands). The sum of squared deviations (SSD), stress (*S*), and the correlation (*r*) values between the original and simulated matrices were used to evaluate the optimal number of polymorphic bands according to Kruskal (1964). *S* values indicate the difference between the original matrix and the simulated matrix. When the value of $S > 0.05$, the number of fragments is considered sufficient for the analysis (Kruskal 1964).

Genetic analysis

The amplified DNA fragments were scored as present (1) or absent (0). Only reproducible bands were scored;

Table 1 Summary of ISSR primers used in the screening of *B. tuberculata*, *C. bicolor*, *C. labiata*, *C. granulosa* and *C. shofieldiana*

ISSR primers	Sequence (5′–3′)	Loci	Polymorphic loci (P_L)	% P_L, 0.95 criterion	Range of amplified bands (bp)	PIC
UBC 807 AG_8T	AGA GAG AGA GAG AGA GT	14	14	100	600–2,000	0.329
UBC 809 AG_8G	AGA GAG AGA GAG AGA GG	17	17	100	600–2,500	0.448
UBC 821 GT_8T	GTG TGT GTG TGT GTG TT	11	11	100	700–2,000	0.334
UBC 822 TC_8A	TCT CTC TCT CTC TCT CA	16	16	100	1,000–8,000	0.245
UBC 826 AC_8C	ACA CAC ACA CAC ACA CC	17	16	94	600–2,500	0.406
UBC 827 AC_8G	ACA CAC ACA CAC ACA CG	20	19	95	600–3,000	0.297
UBC 842 GA_8YG	GAG AGA GAG AGA GAG AYG	16	16	100	300–1,300	0.458
UBC 851 GT_8YG	GTGTGTGTGTGTGTG TYG	12	12	100	600–1,500	0.258
UBC 860 TG_8RA	TGT GTG TGT GTG TGT GRA	13	13	100	600–2,000	0.366
UBC 862 AGC_6	AGC AGC AGC AGC AGC AGC	9	6	66.7	600–1,500	0.274
UBC 898 CA_6RY	CAC ACA CAC ACA RY	21	21	100	500–2,500	0.479
Average		15	14	96.99		0.354
Total		166	161			

smeared and weak bands were excluded. To assess the PIC, we used the formula PIC=$1 - \Sigma p_i^2$, where p_i is the frequency of the *i*th amplified allele. The PIC indicates the ability of each marker to be found in two different states (presence/absence) in two plants chosen randomly from the population.

The genetic diversity parameters for each orchid species were analyzed using POPGENE version 1.32 (Yeh el al. 1999) to estimate: the percentage of polymorphic bands (P_L, 0.95 criterion, Nei 1987), number of alleles (*Na*), Nei's (1973) expected heterozygosity (H_E), and the Shanon index of diversity (H_O). The level of genetic differentiation among species was estimated using the analysis of molecular variance (AMOVA), performed in ARLEQUIN software (Excoffier et al. 2009). The test of significance for AMOVA was carried out with 10,000 permutations, as well as the pairwise F_{ST} values and significance. Principal component analysis (PCA) was performed with STATISTICA 7.0 (StatSoft Inc. 2004) using the genetic distance matrix of the individuals with UPGMA as the grouping algorithm (Sneath and Sokal 1973). Aggregations of plotted individuals reveal sets of genetically similar individuals.

Results

ISSR profiles

The 11 selected ISSR markers generated 166 unambiguous and reproducible bands, 161 (96.7 %) of which were polymorphic, with sizes ranging from 300 to 8,000 bp. The number of bands varied from 9 (UBC 862) to 21 (UBC 898) with an average of 15 bands per ISSR markers (Table 1). Both the primers UBC 898 and UBC 827 presented 21 and 20 ISSR loci for di-nucleotide CA and AC repeats, respectively. This was the most informative result among the tested ISSR primers. The PIC values ranged from 0.245 (UBC 822) to 0.479 (UBC 898) with a mean of 0.354, thus demonstrating the strong discriminatory power of the markers identified (Table 1).

Optimum number of ISSR markers

For all species we found high correlation values ($r > 0.96$) between the original (analyzed bands) and simulated matrices (Table 2), with consistency in 156 bands (ONM) at which point the correlation value reached 0.9952 and stress reached 0.0365 for all species. The results in this study showed that the ONM was lower than the number of loci used (166 bands); from this, it can be inferred that the estimates of genetic diversity detected among individuals of *B. tuberculata*, *C. bicolor*, *C. granulosa*, *C. labiata*, *C. schofieldiana* present excellent accuracy, between 116 and 146 loci ($E < 0.05$) (Fig. 1).

Table 2 Analysis of optimal number of ISSR markers (ONM) for five species of Laeliinae

Species	ONM	Correlation	SQ deviation	Stress
B. tuberculata	146	0.9958	0.0049	0.0424
C. bicolor	136	0.9924	0.0072	0.0476
C. granulosa	141	0.9968	0.0021	0.0495
C. labiata	146	0.9810	0.0021	0.0486
C. schofieldiana	116	0.9661	0.0088	0.0490
All species	156	0.9952	0.0571	0.0365

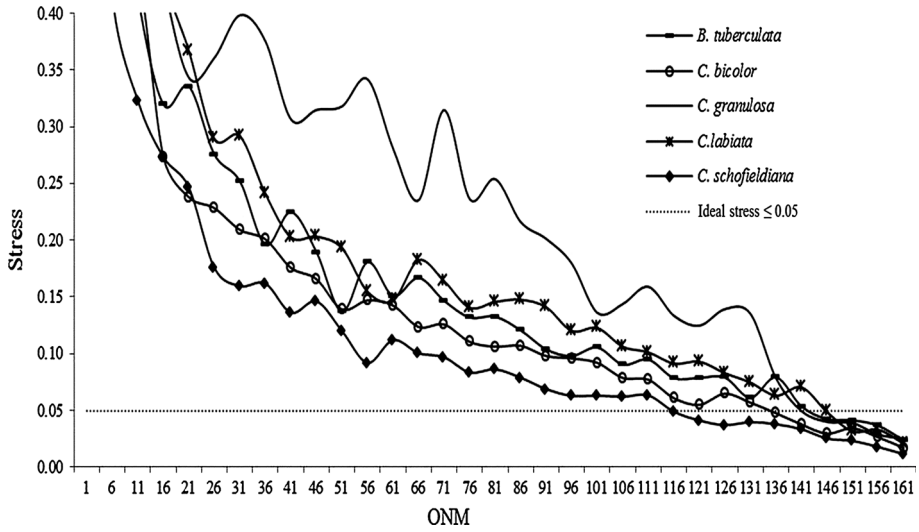

Fig. 1 Optimum number of ISSR markers for *B. tuberculata* (Bt), *C. bicolor* (Cb), *C. granulosa* (Cg), *C. labiata* (Cl), and *C. schofieldiana* (Cs)

Table 3 Measures of genetic diversity of *B. tuberculata* (Bt), *C. bicolor* (Cb), *C. labiata* (Cl), *C. granulosa* (Cg) and *C. schofieldiana* (Cs)

Codes	*n*	*L*	*P*	*Na*	*Ne*	H_E	H_O	F_{ST}
Bt	5	87	52.41	1.524 ± 0.0388	1.387 ± 0.0315	0.216 ± 0.0168	0.314 ± 0.0239	0.214
Cb	5	94	56.63	1.566 ± 0.0386	1.383 ± 0.0303	0.219 ± 0.0161	0.323 ± 0.0230	0.177
Cl	5	50	30.12	1.323 ± 0.0364	1.230 ± 0.0273	0.132 ± 0.0152	0.193 ± 0.0219	0.256
Cg	5	51	30.72	1.395 ± 0.0381	1.292 ± 0.0305	0.163 ± 0.0162	0.237 ± 0.0232	0.322
Cs	5	93	56.02	1.560 ± 0.0386	1.374 ± 0.0326	0.213 ± 0.0162	0.314 ± 0.0230	0.219

Sample size (*n*), polymorphic loci (*L*) and percentage of polymorphic loci $P_{(0.95)}$, number of alleles (*Na*, mean ± SE), effective number of alleles (*Ne*, mean ± SE), gene diversity (H_E, mean ± SE), Shannon index of diversity (H_O, mean ± SE), fixation index (F_{ST})

Genetic diversity

Using the ISSR markers, genetic variation was analyzed within each orchid species (Table 3). *C. bicolor* and *C. schofieldiana* showed the greatest number of polymorphic loci (*L*), with 94 and 93, respectively. The percentage of polymorphic loci (*P*) ranged from 30.12 % in *C. labiata* to 56.63 % in *C. bicolor*. Of the five species, *C. bicolor* exhibited the highest level of variability ($H_E = 0.219$; $H_O = 0.323$), while *C. labiata* exhibited the lowest level ($H_E = 0.132$; $H_O = 0.193$) (Table 3).

Genetic differentiation

According to the AMOVA analysis, the percentage of genetic variation within Laeliinae species was 76.74 %, indicating that genetic differentiation was found mainly within species rather than between species (Table 4). The fixation index showed that the most structured species is *C. granulosa*, with an F_{ST} value of 0.322 (Table 3).

Principal component analysis of all five species sampled showed that species were clearly and unequivocally differentiated along ordination axis 1 (Fig. 2), which captured 26.70 % of the total variance. Axis 2 (36.57 % of the total variance) differentiated the *B. tuberculata* population from the *Cattleya* spp.

Table 4 Analysis of molecular variance (AMOVA) for *B. tuberculata*, *C. bicolor*, *C. granulosa*, *C. labiata* and *C. schofieldiana* based on 166 ISSR markers

Source of variation	*df*	Sum of squares	Variance components	% total of variation	*P*
Among species	4	64.536	2.10536	23.26	0.00030
Within species	17	118.100	6.94706	76.74	0.00001
Total		182.636	9.05242		
Fixation index		Φ_{ST}: 0.232			

df degrees of freedom

Discussion

ISSR markers and genetic variation

Molecular-marker-based studies of genetic diversity in plant species are important tools for plant breeding and

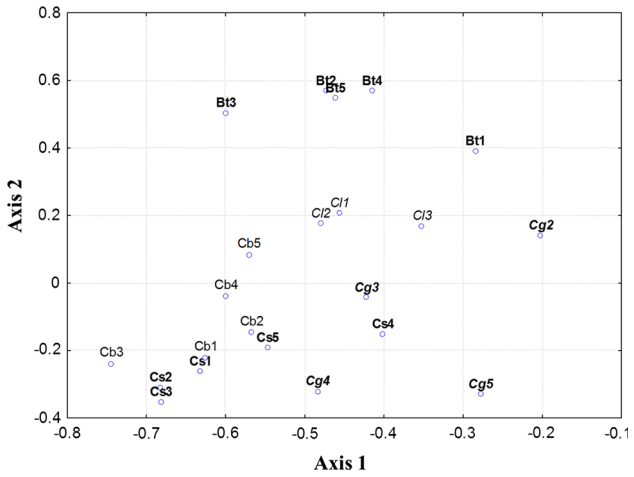

Fig. 2 Principal component analysis of ISSR data for Laeliinae. Percentage of variance accumulated on the axes: axis 1 = 26.70 %; axis 2 = 36.57 %. *B. tuberculata* (Bt), *C. bicolor* (Cb), *C. granulosa* (Cg), *C. labiata* (Cl) and *C. schofieldiana* (Cs)

conservation (Nybom and Bartish 2000). This study represents the first use of the ISSR genotyping technique to measure the genetic variation and assess phylogenetic inferences of five Laeliinae species from the Atlantic Forest of Brazil. The majority of studies on the Laeliinae subtribe focus on the phylogeny of the group using plastid markers and nuclear ITS rDNA (van den Berg et al. 2000, 2009). In the literature, only the analysis of genetic diversity in *C. labiata* and its related genera using RAPD (Jin et al. 2004) has been discussed. Recently, Pinheiro et al. (2012) also evaluated natural populations of *C. labiata* and ten species of the same genus using RAPD and ISSR.

The number of loci used in studies of genetic diversity of plant species, with ISSR molecular markers is variable (e.g., Zhang et al. 2010; Brandão et al. 2011). Verma et al. (2009) selected 11 ISSR primers to analyze genetic diversity of 9 species of *Vanilla*, and found 108 loci of which 93 were polymorphic (86.11 %). Wang et al. (2009b) evaluated the genetic diversity and phylogenetic relationships among 31 *Dendrobium* species with 17 ISSR primers and obtained 278 loci with 100 % polymorphism. According to Colombo et al. (2000), between 50 and 100 bands is considered sufficient to estimate genetic relationships among and within plant species. Thus, we concluded that the ISSR markers used in this study are sufficient not only for quantifying the genetic diversity of *B. tuberculata*, *C. bicolor*, *C. granulosa*, *C. labiata* and *C. schofieldiana*, but also to identify polymorphism between individuals in populations.

The results show that the genetic parameters *Ne*, *He* and *Ho* were statistically identical among *B. tuberculata*, *C. bicolor* and *C. shofieldiana*, as compared with the standard error (Table 3). However, the *Na* parameter was statistically different among the five species. Although the sampling strategy does not permit us to make conclusions about the levels of genetic diversity, long-lived and outcrossing species are expected to be more variable than annual and self-compatible species (Hamrick and Godt 1990). Thus, in future studies, the sampling of populations should consider the geographical distribution of the species, as well as its demographic and reproductive characteristics.

Species characterization, phylogenetic relationships and genetic differentiation

Laeliinae occurs exclusively in the Neotropical region, so it has many peculiarities and ecological morphological traits. The majority of species of the subtribe are epiphytes or rupicolous and have thick leaves and pseudobulbs as an adaptation to xeric habitats (van den Berg et al. 2009).

Unlike *C. labiata*, the other *Cattleya* species assessed in this study, *C. granulosa*, *C. schofieldiana* and *C. bicolor*, are part of the bifoliolate group (Table 5). The genus *Cattleya* is characterized by its large flowers and its lip is not fused to the column. *Cattleya* plants exhibit a strong rootstock, that is slightly reptant, and each growing season, one or two leaf groups root and a new branch is formed. This branch is claviform, flattened laterally, and elongated and thin (bifoliolate species). There are two species groups: the first has spindle-shaped pseudobulbs that are strongly compressed laterally with only one leaf (unifoliate); the second is cylindrical and has clavate pseudobulbs with two bracts (bifoliate), and occasionally present three shoots (Braem 1984). The flower buds are surrounded by one or two bracts, which are called the spathe. The flowers are composed of three petals and sepals and two petals form the lip. The lip structure is much more elaborate than the other parts of the flower and involves the spine (Braem 1994).

Cattleya labiata Lindl. is a unifoliate orchid (Table 5). According to Menezes (2002) it is known as the "*Queen of Northeast Brazil*" because of its beauty, variety of fragrances, and cultivability; the plant is adaptable to different locations and types of substrate. *C. labiata* is characterized as having only one leaf per pseudobulb ranging from 7 to 20 cm long. The flowers range from 10 to 20 cm in length. They are usually pinkish-purple or pinkish-red, but several varieties have been recorded. Analyses using RAPD and ISSR markers found high levels of polymorphism in the *C. labiata* (Pinheiro et al. 2012). The species is endangered (MMA 2008) and suffers from extractivism and habitat loss from deforestation.

Cattleya granulosa varies in size and the plants may have long or short pseudobulbs containing from two to

Table 5 Taxonomic data, geographical distribution, habitat, ploidy (2*n*), and conservation status of Laeliinae species

Species	Leaf	Endemism	Phytogeographic domains	Geographical distribution	Habitat	2*n*	Endangered
B. tuberculata	Unifoliate	No	Caatinga, Cerrado and Atlantic Forest	Argentina, Bolivia, Paraguay, Peru, and Brazil North (TO), Northeast (RN, PB, PE, BA, AL, SE), Southeast (MG, SP, RJ), South (PR, SC, RS).	Epiphyte or rupiculous	40	No
C. bicolor	Bifoliolate	Yes	Cerrado and Atlantic Forest	Southeastern states (SP, RJ, MG, ES), the Midwest (DF)	Epiphyte	40	Yes
C. labiata	Unifoliate	Yes	Atlantic Forest and Caatinga	Northeastern states (CE, PA, PE, AL, SE) and states Southeast (ES RJ)	Epiphyte or rupiculous	40	Yes
C. granulosa	Bifoliolate	Yes	Atlantic Forest	Northeastern states (RN, PA, PE and AL) and Southeast (ES)	Epiphyte or terrestrial	40	Yes
C. schofieldiana	Bifoliolate	Yes	Atlantic Forest	Northeastern states (BA) Southeast states (ES, RJ)	Epiphyte	40	Yes

Barros et al. (2013); Braem (1994); Cunha and Forzza (2007); Menezes (2002); Mora et al. (2008)

Abbreviation of the Brazilian states: Alagoas (AL), Bahia (BA), Ceará (CE), Distrito Federal (DF), Espirito Santo (ES), Minas Gerais (MG), Paraíba (PB), Paraná (PR), Pernambuco (PE), Santa Catarina (SC) Sergipe (SE), São Paulo (SP), Rio de Janeiro (RJ), Rio Grande do Norte (RN), Rio Grande do Sul (RS), Tocantins (TO)

three leaves. The flowers are approximately 15 cm in diameter and vary in number from three to seven per pseudobulb. Flowers range in color from yellow to green to red. *C. granulosa* was included on the official list of endangered flora published by the Brazilian Ministry of the Environment in 2008. This species has suffered significant losses due to the harvesting of specimens and habitat loss (Barros et al. 2013).

Cattleya schofieldiana is endemic to Brazil and it is found in the phytogeographic domain of the Atlantic Forest, occurring in the states of Espírito Santo and Rio de Janeiro. The plants reach 1 m in height and the flowers can reach 10 cm in length and are very fragrant. *C. schofieldiana* produces between two and five flowers per stem, of yellow to dark brown with stains on the petals and sepals. The purple or magenta lip protrusion has many hairs and has a white border (Barros et al. 2013).

Cattleya bicolor is considered a vulnerable species due to several factors, including over-collection and habitat destruction. Of all the species in the genus, *C. bicolor* is the species for which the taxonomy is most often confused. The lateral lobes of the lips are significantly reduced, allowing easy identification within the genus. *C. bicolor* orchid is medium to large in size. The pseudobulbs are long, cylindrical, ribbed, and oblong with two leaves. The petals and sepals of *C. bicolor* feature a mahogany-brown with purple tones, the lip is rosy pink or magenta. The colors of the flowers can vary considerably between individuals. The point of the flower emerges from a basal sheath and emits between one and seven flowers (Mora et al. 2008).

Phylogenetic studies on the Laeliinae subtribe (Epidendroideae, Orchidaceae) suggest important taxonomic changes in recent years (van den Berg et al. 2000, 2009). The first molecular phylogenetic analysis on Laeliineae, which was based on ITS sequences, suggested that the genus is not monophyletic (van den Berg et al. 2000).

The results of our study show that the fixation index for *C. bicolor* ($F_{ST} = 0.177$), *B. tuberculata* ($F_{ST} = 0.214$), and *C. shofieldiana* ($F_{ST} = 0.219$) are similar to the average found for Orchidaceae ($F_{ST} = 0.146$), which is typically characterized by relatively low genetic differentiation among populations (Phillips et al. 2012). Despite the genetic differences observed in this study ($F_{ST} = 23.2$ %), the *Brassavola* group is a close relative of the unifoliate *Cattleya* (e.g., *C. labiata*, see Table 5). *B. tuberculata* often occur on inselbergs, as an epiphyte in the salt marshes of the coast of Brazil, and on rocky outcrops as rupicolous (Cunha and Forzza 2007). This species, also known as the "rat-tail orchid", is characterized by having long arching leaves. Its flowers, with greenish petals and sepals and white lip ranging from four to ten per stem, open in the summer coinciding with the blooming of *C. labiata* (Barros et al. 2013). In fact, PCA showed greater genetic similarity between these two unifoliate species (Fig. 2). Still, according to the PCA, bifoliate species are divergent from unifoliate species, forming a secondary group explained by axis 2. Finally, the data suggest that *C. shofieldiana* and *C. granulosa* are closely related. *C. shofieldiana* is often considered a variety of *C. granulosa* and is known as *Cattleya granulosa* var. *schofieldiana* (Rchb.f.) A.H.Kent (Govaerts 2003; Barros et al. 2013).

The data obtained using ISSR markers to analyze these species have shown that they are effective in assessing interspecific differentiation. Such analyses may contribute to the conservation of Neotropical orchid populations and

species. In fact, phylogenetic relationships are being used increasingly as a tool to study conservation genetics. In such analyses, one of the most important applications is in the definition of taxonomic units (Allendorf and Luikart 2007). Thus, the results of this study can be applied to studies of population genetics in order to define priority areas for the conservation of orchids in their remaining natural habitats.

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