Genetics and Molecular Research

Analysis of genetic diversity of Laeliinae (Orchidaceae) in the State of Sergipe using ISSR markers

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ABSTRACT. The Orchidaceae represent one of the largest and most diverse families on the planet. However, this family is constantly threatened by predators and by the advancement of urban centers over its natural habitats. The objective of this study was to use inter-simple sequence repeat markers to evaluate the genetic diversity between orchid accessions of the Laeliinae subtribe, which comprise part of the Orchidaceae study collection at the Department of Agronomic Engineering of the Federal University of Sergipe. DNA was extracted from each specimen by using an adapted 2% cetyltrimethyl ammonium bromide protocol. Similarity between individuals was calculated using the Jaccard method. Clustering was carried out by the unweighted pair group method with arithmetic mean method, with resampling and 10,000 bootstraps. Eighty-seven fragments were obtained, all of which were polymorphic, revealing high variability between accessions. The mean similarity was 35.77% between *Encyclia* sp individuals,

and 35.90% between specimens of *Cattleya tigrina*. For *Epidendrum secundum*, a relationship between geographic and genetic distances was observed, and the accession collected in the southern part of the State of Sergipe (Serra de Itabaiana National Park) was more divergent than that of the other parts of the state. The data generated in this study will guide further research aimed at the *ex situ* conservation of these materials.

Key words: *Cattleya tigrina*; Conservation; Genetic diversity; *Encyclia* sp; *Epidendrum* sp; ISSR

INTRODUCTION

The orchid family contains about 35,000 species that are distributed in over 1800 genera (Watanabe, 2002) and are dispersed throughout the world, from the polar regions to equatorial deserts (Moreira et al., 2007), with greater diversity in the tropical region. This family represents 7% of all species on the planet (Altafin et al., 2003; Rech et al., 2011), and is considered by many botanists to be the most evolved of the Liliopsida (Raven et al., 2007). However, Orchidaceae is also considered to be the family with the highest percentage of endangered genera and species.

Destruction of native vegetation and the resulting loss of genetic diversity account for the vulnerability of these species. The loss of genetic variability may reduce the ability of a species to adapt to biotic and abiotic changes, intensifying the extinction process. Studies on patterns of genetic diversity within populations and the structure between populations may help to infer evolutionary mechanisms, such as genetic drift, selection, and mutation. Furthermore, the spatial genetic structure may serve as an indicator of the extent of gene flow and population divergence (George et al., 2009).

Molecular markers are used widely in studies on genetic diversity. Inter-simple sequence repeat (ISSR) markers have been used extensively in studies of diversity and genetic structure of many species, because compared to other markers, ISSR markers are more reproducible, stabler, simpler, and easier to work with (George et al., 2009; Fang et al., 2012).

Some studies involving the Orchidaceae family have evaluated the genetic diversity and conservation of native populations, such as the use of ISSR markers in the investigation of diversity in *Piperia yadonii* allozymes and *Goodyera rosulacea*, both of which revealed low variation between individuals and highlighted the immediate need for development of conservation strategies (George et al., 2009; Chung and Chung, 2010). In addition, random amplified polymorphic DNA (RAPD) and ISSR markers have been used in studies on *Cymbidium* sp (Sharma et al., 2011), and microsatellite markers have been used to study *Dendrobium loddigesii* (Cai et al., 2012).

Studies using ISSR and RAPD markers to investigate diversity in populations of *Cattleya labiata* from northeastern Brazil found 272 fragments, Jaccard similarity coefficients ranging from 0.14 to 0.82, and mean similarity of 50% within populations. Although this variation is relatively high, the authors note that these populations are isolated owing to the fragmentation of the main habitat of the species (Atlantic Forest). Under these circumstances, there is a risk of losing some populations, and consequently, some alleles and characteristics that are not shared by individuals from other clusters. This limits the ability of preserving this species (Pinheiro et al., 2012).

The objective of this article was to study the genetic diversity conserved within the

Laeliinae subtribe in the State of Sergipe. The subtribe is represented by the genera *Encyclia* Hook, *Epidendrum* L., and *Cattleya* Lindl.

MATERIAL AND METHODS

Plant material

Based on inventories and surveys of the flora of Sergipe, a biogeographic study was carried out on orchid populations in the state. Next, germplasm collection expeditions were carried out in the regions where these populations occur, and random sampling was carried out in these locations, as well as mapping, with the aid of a Global Positioning System (GPS) (GARMIN, Schaffhausen, Switzerland).

Exploited areas were remnants of the Atlantic Forest in the State of Sergipe, and represent the main biome in which the Orchidaceae family occurs within the state. In total, 143 individuals were collected in eight different regions.

Individuals were coded, planted in ceramic pots containing carbonized wood chips and pine bark (1:1), and kept in a greenhouse with regular watering. Species of collected accessions were identified using the taxonomic key in the ASE Herbarium of the Federal University of Sergipe (UFS).

Of the collected individuals, 31 were identified to the genus level (15 different botanical genera), and 45 to the species level. Of these, 30 individuals of the Laeliinae subtribe (13 *Encyclia*, 12 *Epidendrum*, and 5 *Cattleya*) (Table 1) were used to study diversity.

Table 1. Orchidaceae collection of the Universidade Federal de Sergipe, collected in municipalities of the State of Sergipe (Brazil).

Name	Code	Municipality of origin	Scientific name	Geographic data
RD_Cat.tigrina	ORQ 162	Riachão do Dantas	*Cattleya tigrina*	11°04'30.9"S 37°49'46.2"W
RD_Cat.tigrina1	ORQ 166	Riachão do Dantas	*Cattleya tigrina*	11°04'33.4"S 37°49'47.9"W
RD_Cat.tigrina2	ORQ 174	Riachão do Dantas	*Cattleya tigrina*	11°04'36.4"S 37°49'44.2"W
RD_Cat.tigrina3	ORQ 175	Riachão do Dantas	*Cattleya tigrina*	11°04'36.5"S 37°49'43.3"W
RD_Cat.tigrina4	ORQ 179	Riachão do Dantas	*Cattleya tigrina*	11°04'36.7"S 37°49"45.0"W
RI_Encyclia	ORQ 120	Ribeira	*Encyclia dichroma*	10°49'33.0"S 37°26"24.0"W
SI_Encyclia	ORQ 017	Serra de Itabaiana	*Encyclia dichroma*	10°45'26.6"S 37°20'29.9"W
SI_Encyclia2	ORQ 022	Serra de Itabaiana	*Encyclia dichroma*	10°45'31.8"S 37°20'29.4"W
RI_Encyclia2	ORQ 088	Ribeira	*Encyclia* sp	10°49'33.0"S 37°26'24.0"W
RdasP_Encyclia	ORQ 126	Rio das Pedras	*Encyclia* sp	10°47'18.0"S 37°25'47.1"W
RdasP_Encyclia2	ORQ 127	Rio das Pedras	*Encyclia* sp	10°47'18.0"S 37°25'47.1"W
RdasP_Encyclia3	ORQ 128	Rio das Pedras	*Encyclia* sp	10°47'18.0"S 37°25'47.1"W
RdasP_Encyclia4	ORQ 132	Rio das Pedras	*Encyclia* sp	10°47'18.0"S 37°25'47.6"W
SI_Encyclia3	ORQ 035	Serra de Itabaiana	*Encyclia* sp	10°45'07.0"S 37°21'57.0"W
SI_Encyclia4	ORQ 037	Serra de Itabaiana	*Encyclia* sp	10°45'10.6"S 37°21'59.6"W
SI_Encyclia5	ORQ 039	Serra de Itabaiana	*Encyclia* sp	10°45'10.6"S 37°21'59.6"W
SI_Encyclia6	ORQ 043	Serra de Itabaiana	*Encyclia* sp	10°45'25.9"S 37°22'05.6"W
SI_Encyclia7	ORQ 054	Serra de Itabaiana	*Encyclia* sp	10°45'09.3"S 37°21'58.0"W
SI_Encyclia8	ORQ 057	Serra de Itabaiana	*Encyclia* sp	10°45'07.0"S 37°21'57.0"W
SI_Encyclia9	ORQ 058	Serra de Itabaiana	*Encyclia* sp	10°45'32.0"S 37°20'30.7"W
RdasP_E.secudum	ORQ 137	Rio das Pedras	*Epidendrum secundum*	10°47'18.0"S 37°25'47.6"W
RdasP_E.secudum2	ORQ 141	Rio das Pedras	*Epidendrum secundum*	10°47'18.0"S 37°25'47.6"W
TG_E.secudum	ORQ 046	Serra de Itabaiana	*Epidendrum secundum*	10°45'20.5"S 37°22'06.4"W
SI_E.secudum2	ORQ 008	Serra de Itabaiana	*Epidendrum* sp	10°45'39.7"S 37°20'24.7"W
SI_E.secudum3	ORQ 020	Serra de Itabaiana	*Epidendrum* sp	10°45'30.3"S 37°20'30.7"W
SI_E.secudum4	ORQ 024	Serra de Itabaiana	*Epidendrum* sp	10°45'31.8"S 37°20'29.4"W
SI_E.secudum5	ORQ 055	Serra de Itabaiana	*Epidendrum* sp	10°45'07.1"S 37°21'57.0"W
SI_E.secudum7	ORQ 062	Serra de Itabaiana	*Epidendrum* sp	10°44'57.3"S 37°21'04.2"W
X_E.059	ORQ 059	Serra de Itabaiana	*Epidendrum* sp	10°45'07.0"S 37°21'57.0"W
X_E.116	ORQ 116	Serra de Itabaiana	*Epidendrum* sp	10°44'57.3"S 37°21'04.2"W

SSR markers

Young leaves were collected from the UFS collection and submitted to an adapted DNA extraction method using 2% cetyltrimethyl ammonium bromide (CTAB) (Doyle, 1991). About 1 g young leaf was macerated in a mortar with 10 mL 2% CTAB buffer. Reactions were carried out at the Molecular Genetics Laboratory of Department of Biology, Federal University of Lavras, UFLA (Brazil). The total reaction volume was 12 mL, which contained 2.25 µL genomic DNA (10 ng/µL), 2.25 µL primer, 1.0 mL buffer, 0.66 µL dNTP (10 mM), 0.6 µL Taq DNA polymerase, and 5.25 mL ultrapure water. Eight primers were used (Table 2).

Table 2. Sequences and annealing temperature of inter simple sequence repeat (ISSR) primers used to characterize orchids collected in municipalities of the State of Sergipe (Brazil).

Primer	Sequence (5'-3')	Annealing temperature (°C)
ISSR 3	TG GA TG GA TG GA TG GA	55
ISSR 6	AC TG AC TG AC TG AC TG	55
ISSR 7	GTG GTG GTG GTG GTG	55
ISSR 9	AC AC AC AC AC AC AC AC-CG	55
ISSR 15	CA CA CA CA CA CA CA CA-AG	50
ISSR 813	CTC TCT CTC TCT CTC TT	55
ISSR 827	ACA CAC ACA CAC ACA CG	48
ISSR 848	CAC ACA CAC ACA CAC ARG	50

Amplification was carried out using 1.5% agarose gel with 4 mL per 100 mL RED® gel (Biotium, Hayward, CA, USA), run at 90 V for 3 h. Amplified products were visualized and photographed under ultraviolet light in a KODAK EDAS 290 photo documentation system.

Statistical and genetic analyses

Electrophoretic profiles of ISSR markers were transformed into a binary matrix. The presence of a fragment in a particular individual was represented by 1, and the absence of the same fragment was represented by 0. Similarity was calculated using the Jaccard method. Accessions were clustered based on similarity, using the unweighted pair group method with arithmetic mean (UPGMA). To evaluate the robustness of clustering between genotypes, bootstrap resampling was carried out with the same size of the original sample. For each resampling level, 10,000 bootstrap samples were obtained. This analysis was carried out using the FreeTree software (Pavlicek et al., 1999). The dendrogram was obtained by the TreeView software (Page, 1996).

RESULTS

The eight ISSR primers produced a total of 87 fragments, all of which were polymorphic, with a mean ~11 fragments per primer. The highest number of bands was obtained by ISSR 7 (17 fragments), and the lowest number of bands was obtained by ISSR 6 (7 fragments).

Similarity between individuals of the *Encyclia* genus (Table 3) ranged from 4% (between individuals collected, in the town of Rio das Pedras - municipality of Itabaiana, and another, in the Serra de Itabaiana National Park) to 76% (between individuals of the National Park). The mean similarity for this genus was 35.77%.

Table 3. Jaccard similarity (%) of individuals of the genus *Encyclia* Hook from the Orchidaceae collection of the Universidade Federal de Sergipe, collected in municipalities of the State of Sergipe (Brazil).

	SI_ En	SI_En2	SD_En	RI_En	SI_En3	SI_En4	SI_En5	SI_En6	SI_En7	RP_En	SI_En8	RP_En2	SI_En9
SI_En													
SI_En2	56												
SD_En	43	39											
RI_En	36	49	43										
SI_En3	35	37	64	49									
SI_En4	44	40	54	53	62								
SI_En5	17	28	22	37	32	30							
SI_En6	28	30	37	56	43	61	44						
SI_En7	38	35	43	58	56	71	40	76					
RP_En	19	26	37	47	43	48	24	52	54				
SI_En8	20	15	12	16	11	20	10	14	20	04			
RP_En2	32	30	28	35	30	37	33	41	42	31	14		
SI_En9	30	28	26	30	32	40	26	29	35	24	21	38	

*Individuals preceded by SI were collected in the Serra de Itabaiana National Park; individuals preceded by SD were collected in Simão Dias; individuals preceded by RI were collected in Ribeira; and individuals preceded by RP were collected in the village of Rio das Pedras, municipality of Itabaiana, State of Sergipe.

Clustering of *Encyclia* accessions revealed that the most divergent was specimen 8 collected in the Serra de Itabaiana National Park (SI_Encyclia8). The two most closely related specimens were collected in the park (SI_Encyclia and SI_ Encyclia2) (78%) (Figure 1). For *Epidendrum* L., the lowest degree of similarity (12%) was found between *E. secundum* individuals collected in the municipality of Tomar do Geru and in the Serra de Itabaiana National Park (south and central regions of the state, respectively) (Table 4). SI_E. secundum, SI_E. secundum2, and SI_E. secundum3 presented 100% similarity by resampling (bootstrap). SI_E. secundum4, SI_E. secundum5, SI_E. secundum6, RdasP_E.secundum, and RdasP_E. secundum2 formed the second cluster, with 83% similarity (Figure 2).

The *Cattleya* Lindley genus is only represented in the present collection by *Cattleya tigrina* A. Rich., although the occurrence of *Cattleya labiata* Lindl. has also been confirmed in the State of Sergipe (Monteiro et al., 2012a). Table 5 shows the similarity between the five specimens of the species *Cattleya tigrina* A. Rich of the studied collection. The genetic similarity ranged from 16 to 71% for *Cattleya tigrina*, with a mean similarity of 35.9%. However, since the sample was limited, it is difficult to evaluate the real conservation status of the population, which is so far the only report on the species in the State of Sergipe.

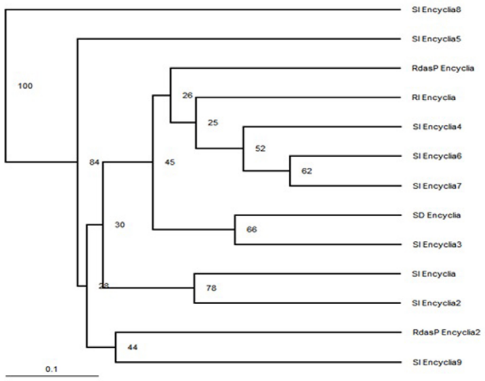

Figure 1. Unweighted pair group method with arithmetic mean (UPGMA) clustering of accessions of the genus *Encyclia* Hook from the Orchidaceae collection of the Federal University of Sergipe.

Table 4. Jaccard similarity (%) of individuals of the genus *Epidendrum* from the Orchidaceae collection of the Universidade Federal de Sergipe, collected in municipalities of the State of Sergipe (Brazil).

	SI_E.s.	SI_E.s2	SI_E.s3	SI_E.s4	SI_E.s5	SI_E.s6	RP_E.s	RP_E.s2	TG_E.s	E.noc.	SI_E.059	E.116
SI_E.s												
SI_E.s2	77											
SI_E.s3	68	74										
SI_E.s4	28	36	34									
SI_E.s5	36	37	34	55								
SI_E.s6	24	28	29	59	42							
RP_E.s	36	41	43	64	46	59						
RP_E.s2	28	32	30	69	43	68	68					
TG_E.s	12	21	22	26	25	29	28	33				
E.noc.	30	27	33	32	32	34	27	35	21			
SI_E.059	29	30	37	32	31	37	29	34	24	87		
E.116	31	28	26	14	18	23	24	23	25	28	27	

*Individuals preceded by SI were collected in Serra de Itabaiana National Park; individuals preceded by TG were collected in Tomar do Geru; and individuals preceded by RP were collected in the town of Rio das Pedras-Itabaiana, SE.

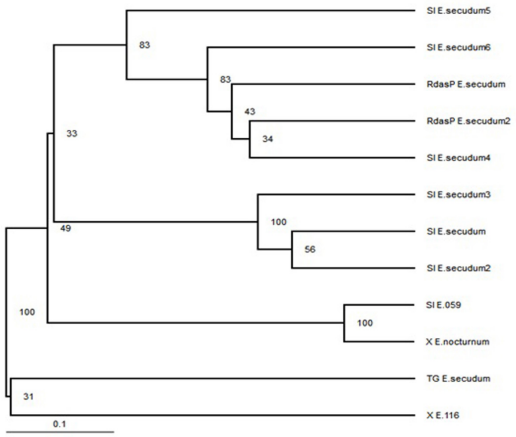

Figure 2. UPGMA clustering of accessions of the genus *Epidendrum* L. from the Orchidaceae collection of the Federal University of Sergipe.

By the resampling method (bootstrap), for instance, RD_Cat.tigrina3, RD_Cat.tigrina4, and RD_Cat.tigrina5 comprise a subcluster with 96% similarity among them and 72% similarity with RD_Cat.tigrina2 (Figure 3).

Table 5. Jaccard similarity (%) of individuals of *Cattleya tigrina* from the Orchidaceae collection of the Universidade Federal de Sergipe, collected in municipalities of the State of Sergipe (Brazil).

	RD_Cat.tigrina	RD_Cat.tigrina2	RD_Cat.tigrina3	RD_Cat.tigrina4	RD_Cat.tigrina5
RD_Cat.tigrina					
RD_Cat.tigrina2	23				
RD_Cat.tigrina3	19	35			
RD_Cat.tigrina4	26	30	71		
RD_Cat.tigrina5	16	27	60	52	

*Individuals preceded by RD were collected in the municipality of Riachão de Dantas, State of Sergipe.

The joint analysis of *Encyclia*, *Epidendrum*, and *Cattleya* confirmed what was observed in the genus separately (Figure 4). Clustering was carried out primarily by genus/species, regardless of the origin of the accessions. *Epidendrum* and *Encyclia* formed a cluster,

which was divided into five subclusters. Ten of the 13 specimens of *Encyclia* comprised a subcluster. Another subcluster was formed by five *E. secundum* individuals, and the least similar was *E. nocturnum* and SI_E.059. RdasP_Encyclia2, SI_Encyclia9, SI_E.secundum, SI_E.secundum2, and SI_E.secundum3 made up the third subcluster. TG_E.secundum and E116 represent the fourth subcluster, and SI_Encyclia8, the fifth subcluster (Figure 4).

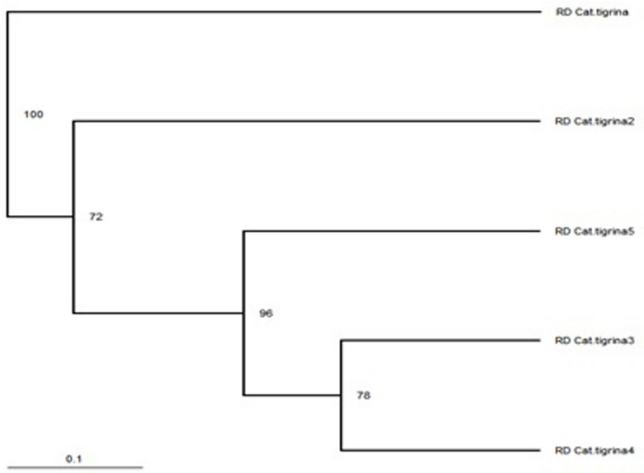

Figure 3. UPGMA clustering of accessions of *Cattleya tigrina* A. Rich. from the Orchidaceae collection of the Federal University of Sergipe.

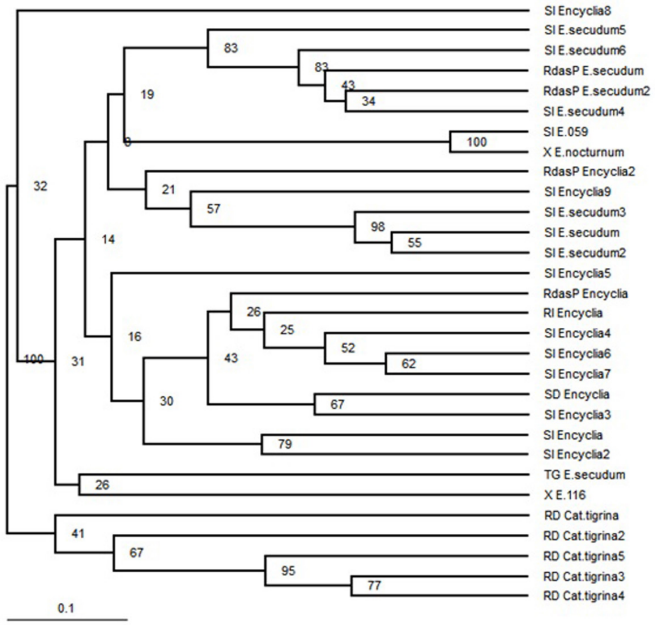

Figure 4. UPGMA clustering of the Laeliinae subtribe from the Orchidaceae collection of the Federal University of Sergipe.

DISCUSSION

Several studies have been carried out using ISSR markers in orchid species. In *Cymbidium goeringii*, 25 ISSR primers produced a total of 210 polymorphic fragments, with a mean number of 8.4 fragments per primer (Wang et al., 2009). In *Cattleya labiata*, a mean number of 12.6 polymorphic fragments per ISSR primer was obtained (151 fragments produced by 12 primers), whereas 11 fragments per primer were obtained using RAPD markers in the same species. The results obtained in this study were close to those obtained with the same type of marker in Orchidaceae individuals (Pinheiro et al., 2012).

The highest and lowest similarity observed between individuals studied in the present article was independent of the location from which they were collected, suggesting that environmental variation does not significantly influence diversity (Figure 1).

Greater genetic similarity was observed between *E. nocturnum* individuals and an individual whose species has yet to be confirmed (SI_E059). The high similarity (87%) suggests that SI_E059, which was collected in the Serra de Itabaiana National Park, is also a specimen of *E. nocturnum*. Another factor that supports this conclusion is the high level of similarity found between SI_E059 and *E. secundum* in this study (37%) (Table 4).

The collected accessions of *Epidendrum* formed four clusters, and unlike *Encyclia*, specimens collected from close locations were found to be more genetically similar.

Confirming the results observed in the similarity table (Table 4), bootstrap analysis showed that SI_E059 (with 100% similarity) clustered with *E. nocturnum*, which appears in the figure preceded by an "X", as it was donated to the collection with no origin identification. Confirmation of the identity of SI_E059 will only be possible after flowering, which will allow it to be classified based on morphological characteristics. TG_E.secundum, collected in the southern part of the Sergipe State (municipality of Tomar do Geru) and E.116 (Serra de Itabaiana National Park) were the most distinct, sharing 31% similarity.

There is more to be learned about Brazilian genetic resources. Most studies performed in the northeast of Brazil, particularly those involving the Orchidaceae family, have involved inventories, floristic surveys, and taxonomic classification.

A study that surveyed and characterized the genus *Catasetum* in the State of Bahia provided a description of the taxa, identification keys, and illustrations of the six identified species (Bastos and Van Den Berg, 2012). A survey on Orchidaceae carried out in the State of Sergipe resulted in a list of 63 species, 34 of which were new records for the state (Monteiro et al., 2012a). Inventories carried out in Sergipe territory also served to include the state in the distribution map of some species, such as *Encyclia alboxanthina* Fowlie (Monteiro et al., 2012b).

The population of *C. labiata* found in the city of Poço Redondo - SE presented Jaccard similarities ranging from 29 to 74%, with a mean value of 56% (Pinheiro et al., 2012). Those authors also found mean similarities of 40-60% in other populations of the States of Pernambuco, Paraíba, and Ceará. Therefore, although they are isolated, populations conserve high intra-population variability, and this knowledge serves to define both the conservation strategy and the choice of individuals selected for improvement. The Laeliinae individuals studied in this article were found to have high genetic variation, especially between genera. Among the *Encyclia* specimen studied, SI_Encyclia8 was the least similar to the others. TG_E. secundum, collected in the southern state, presents less than 30% similarity with individuals of the same species collected in Serra de Itabaiana, and, therefore, is recommended for conservation purposes. Among *Cattleya tigrina* specimens, RD_Cat.tigrina differs from the others and should also be prioritized for conservation.

Conflicts of interest

The authors declare no conflict of interest.

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