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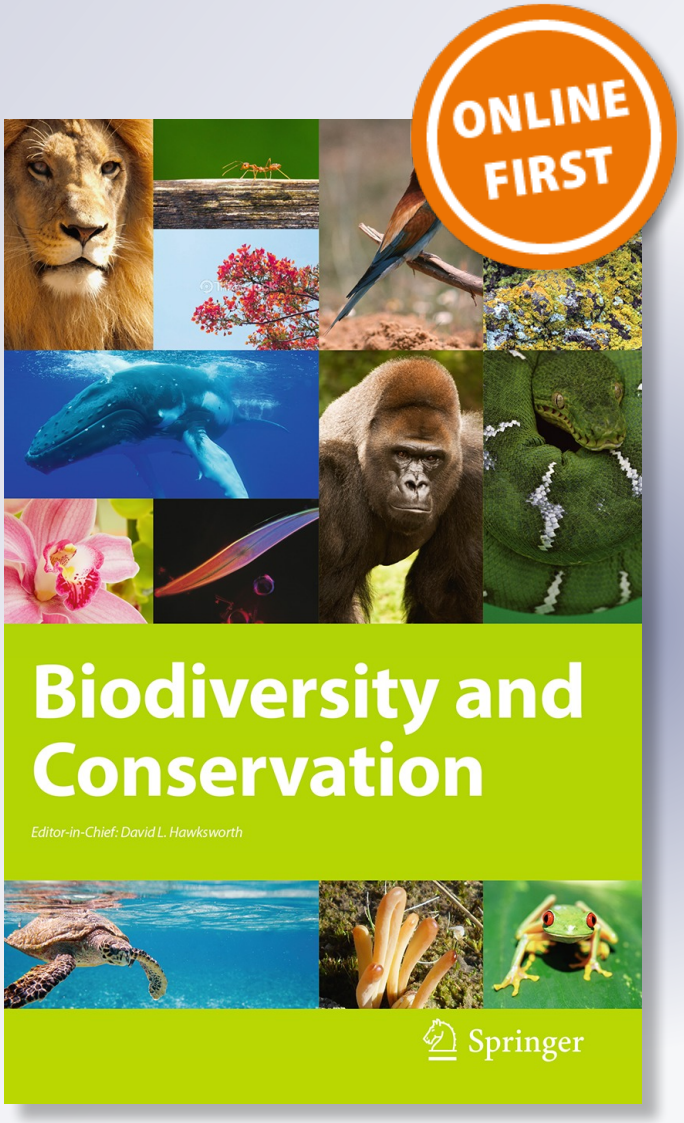

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Negligence in the Atlantic forest, northern Brazil: a case study of an endangered orchid

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Abstract Currently, many Brazilian orchids are threatened with extinction resulting from habitat loss and intense harvesting pressure stemming from their value as ornamental plants. Therefore, the genetic diversity in remaining populations is fundamental to the survival of these species in natural environments. In order to inform conservation strategies, this study evaluated the genetic diversity and structure of *Cattleya granulosa* populations. The sample consisted of 151 individuals from 12 populations in the Atlantic Forest, northeastern Brazil, evaluated using 91 ISSR markers. Genetic variability was assessed through molecular variance, diversity indexes, clusters of genotypes through Bayesian analysis, and tests for genetic bottlenecks. From all polymorphic loci, genetic diversity (H_E) varied between 0.210 and 0.321 and the Shannon index ranged from 0.323 and 0.472. Significant genetic differentiation between populations ($\Phi_{ST} = 0.391$; $P < 0.0001$) resulted in the division of the populations into five groups based on the log-likelihood Bayesian analysis. We found significant positive correlation between geographical and genetic distances between populations ($r = 0.794$; $P = 0.017$), indicating isolation by distance. Patterns of allelic diversity within populations suggest the occurrence of bottlenecks in most *C. granulosa* populations ($n = 8$). Therefore, in order to maintain

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the genetic diversity of the species, the conservation of spatially distant groups is necessary.

Keywords Orchidaceae · Genetic diversity · Vulnerable species · Genetic bottleneck

Introduction

The Orchidaceae family is highly diverse and has a wide range of reproductive strategies, which has resulted in an extensive variety of patterns of genetic differentiation between populations (Wallace 2003; Niknejad et al. 2009). Orchidaceae are successfully adaptive and have phenotypic plasticity; they can adapt to and colonize different niches, even in the most remote regions of the planet, such as the islands of Antarctica (Chase et al. 2003), though there are no marine or aquatic orchids. Compared to other plant families, a large proportion of Orchidaceae genera and species are in danger of extinction (Swarts and Dixon 2009; Martinelli and Moraes 2013). The fragmentation of native forests and illegal extraction have resulted in local extinction of many orchid species (Soto Arenas et al. 2007). Currently, the family is one of the clearest examples of species extinction resulting from human activities (Ávila-Diaz and Oyama 2007). As such, there is an urgent need for more extensive and directed studies on Orchidaceae.

The genus *Cattleya* is exclusively Neotropical, with a wide distribution in Brazil. It includes up to 114 species (Van den Berg et al. 2009, 2014). *Cattleya granulosa* is an endangered epiphytic orchid. While its distribution remains contested, it has been reported mainly in Rio Grande do Norte State, but also in Pernambuco, Alagoas, Paraiba, Bahia, and Espírito Santo States (Cruz et al. 2003). Recent studies indicate its occurrence in Rio Grande do Norte, Paraíba, and Bahia (Martinelli and Moraes 2013), mainly on the coast in restinga forests. The species is threatened by habitat fragmentation, primarily the result of urban development, and predation due to its value as an ornamental plant. Because of these factors, the species is listed as Vulnerable (VU) based on the criteria of the International Union for Conservation of Nature, IUCN (MMA 2008; Martinelli and Moraes 2013).

Significant efforts have been made over the past three decades to obtain information about the genetic variability of orchids; however, considering the diversity of orchid species, the information collected to date are still insufficient, especially for Brazilian species and those threatened with extinction (Borba et al. 2007; Cruz et al. 2011). The existing population genetics studies on orchids have generally focused on terrestrial plants, although there are some epiphytic orchid studies (Parab and Krishnan 2008; Parab et al. 2008; Trapnell et al. 2013). Despite their high species diversity and ecological importance, epiphytic species have received less attention, probably because of difficulties accessing the canopy and the metapopulation structure (Trapnell et al. 2004; Trapnell and Hamrick 2005). However, the limited studies available have shown that outcrossing species have higher genetic diversity than selfing and apomictic species (Sun and Wong 2001; Wallace 2004); geographically widespread species generally have restricted distribution and higher levels of genetic variation than native species (Case et al. 1998; Borba et al. 2001); and large populations tend to have higher genetic diversity than smaller populations (Gustafsson 2000; Cozzolino et al. 2003).

The conservation of genetic diversity is necessary in order to maintain the evolutionary ability of a species to respond to environmental changes and maintain the long term

viability of populations (Toro and Caballero 2005). Population genetic parameters can be estimated from the data obtained with the use of molecular markers based on amplification of the DNA sequence (e.g. inter simple sequence repeats, ISSR), allowing the analysis of genetic diversity and structure and providing information on the dynamics of the alleles in these populations (Fajardo et al. 2014; Rodrigues et al. 2015; Ueno et al. 2015). The genetic structure of a population is shaped by the interaction of evolutionary factors such as mutation, selection, migration, and genetic drift, and influenced by population size, life cycle of the species, and gene flow (Couvet 2002). This final factor depends on the efficiency of dispersers and pollinators to reach other populations, both of which are affected by insulation between populations (Loveless and Hamrick 1984; Nybom and Bartish 2000).

Given the demographic profile of *C. granulosa*, we hypothesized that current populations have experienced recent genetic bottlenecks, with significant decreases in the effective population size. Additionally, in light of the theoretical predictions discussed above, we expect low levels of genetic variation within populations and high levels of genetic differentiation among populations, because of both genetic drift and the limited dispersal of genes, which have a significant impact on small populations (Couvet 2002; Aguilar et al. 2008). Thus, the objective of this study was to describe the levels of intra- and inter-population diversity and the spatial distribution of genetic variability, in order to verify the existence of isolation by distance and define specific groups for *C. granulosa* conservation. We also assessed the occurrence of recent and severe reductions in effective population size (bottlenecks) to identify populations with increased risk of extinction due to the expected loss of genetic variation and consequent loss of adaptive potential.

Materials and methods

Target species

Cattleya granulosa, popularly known as "*canela de ema*" is a perennial epiphyte. It has rhizomatous stems with erect cylindrical pseudo bulbs, that can reach 40–60 cm (Costa 2010). Its flowers are bilaterally symmetrical, with three fleshy petaloid sepals (a dorsal and two sides), ranging in color from greenish yellow to reddish brown, and in most cases presenting granular patches of the color vinacea (Camara-Neto et al. 2007). The flowers are characterized by the presence of osmophores located on the inside of the lateral lobes that exude a heavily sweet odor, as well as nectar guides on the lip that absorb ultraviolet light. The orchid mimics the general model of typical melitophilous flowers, but it produces little floral nectar, likely indicating a deception mechanism with negative frequency-dependent selection (Costa 2010). Additionally, *C. granulosa* is self-compatible and dependent on insects (Euglossini) for sexual reproduction, which are very rare thus resulting in a low frequency of fructification (Costa 2010). This same pattern of self-compatibility and rarity of pollination in natural environments, which is often determined by restrictions on pollinators, have also been observed in other species of Laeliinae (Borba and Braga 2003; Silva-Pereira et al. 2007; Pansarin and Amaral 2008; Vale et al. 2013).

Study sites and sampling

Sampling of *C. granulosa* individuals was performed in small, disconnected remnants of semideciduous and restinga forests in the Atlantic Forest biome (Velozo et al. 1991), northeastern Brazil. We sampled 151 *C. granulosa* individuals (Table 1) from 12 natural populations in the states of Rio Grande do Norte, Paraíba, and Pernambuco (Fig. 1). The populations ESC and MUR have the farthest distance between them, at 303.2 km, and GEN1 and GEN2 populations are the closest with 1.1 km between them. Our sampling strategy aimed to achieve good representation of the population, following methods employed previously for other species of the family (Chung 2009). Populations were georeferenced by GPS, but because *C. granulosa* is an endangered species, we provide only partial coordinates (Table 1). Samples of young and healthy leaves were collected, placed in 1.0 mL tubes containing of 2% CTAB (cetyltrimethylammonium bromide), and stored in a freezer at −20 °C until DNA extraction.

DNA extraction and amplification of ISSR

We used the method described by Doyle and Doyle (1987) to extract DNA. The DNA quantification was assessed by electrophoresis and visualization on 0.8% agarose gels. The DNA amplification reactions were carried out in a Techne thermal cycler TC 412. The reaction consisted of 2 ng of DNA added to 10 μL of the following: 10× PCR buffer (500 mM KCl, 100 mM Tris–HCl pH 8.4; 1% Triton X-100, 20 mM $MgCl_2$); 2.5 mM dNTP; 1 U Taq polymerase; 2 μM primer; and ultrapure water to complete the volume. Thirty-seven amplification cycles were carried out, consisting of an initial step of 2 min at 94 °C, followed by denaturation at 94 °C for 15 s, annealing of the primer to the template DNA ISSR (pairing) at 42 or 47 °C for 30 s, and extension at 72 °C for 1 min. We included a final extension at 72 °C for 7 min.

The amplification products were subjected to electrophoresis on 1.5% agarose gel stained with GelRed™ in 1 X TAE (Tris–Acetate-EDTA). Subsequently, the gels were

Table 1 Geographic location of the populations of *Cattleya granulosa*, population code (Abbr.), geographic coordinates, number of individuals, sample size, and altitude

Municipality (state)	Population	Abbr.	Coordinates	*n*	Altitude (m)
Ceará-Mirim (RN)	Muriú	MUR	5°35′S/35°16′W	18	41
Extremoz (RN)	APA Genipabu1	GEN1	5°42′S/35°12′W	12	40
Extremoz (RN)	APA Genipabu2	GEN2	5°42′S/35°13′W	13	33
Natal (RN)	Parque das Dunas N	PER	5°48′S/35°11′W	13	86
Natal (RN)	Parque das Dunas S	CAR	5°49′S/35°11′W	22	82
Natal (RN)	Parque Dom Nivaldo	NIV	5°50′S/35°13′W	10	77.2
Parnamirim (RN)	Barreira do Inferno	BAR	5°55′S/35°10′W	10	16
Parnamirim (RN)	Alcaçuz	ALC	5°59′S/35°08′W	10	57
Mataraca (PB)	Mataraca	MAT	6°29′S/34°58′W	11	69
Mamanguape (PB)	Mamanguape	PAB	6°52′S/35°06′W	14	11
Cabo de Santo Agostinho (PE)	Cabo de Santo Agostinho	SAN	8°16′S/34°57′W	10	26
Escada (PE)	Escada	ESC	8°20′S/35°11′W	6	177

RN Rio Grande do Norte, *PB* Paraíba, *PE* Pernambuco

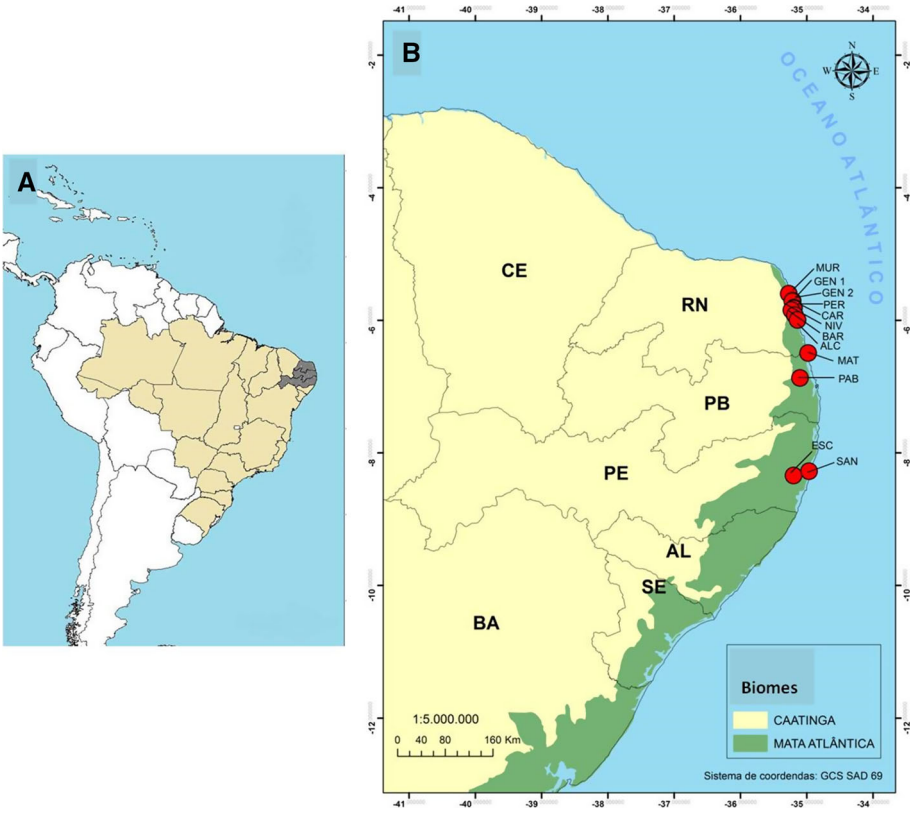

Fig. 1 Geographical location of the sampling areas of *Cattleya granulosa*. **a** Map of Brazil with collection regions highlighted in *gray*. **b** Sampled populations of *Cattleya granulosa* in the states of Rio Grande do Norte (RN), Paraíba (PB), and Pernambuco (PE) within the Atlantic forest biome

photographed under UV light. DNA marker (ladder) of 1 kb was used to estimate the molecular size of the amplified fragments. Twenty primers were tested to identify those that had the best amplification profile. For this, four DNA samples were randomly chosen from the total sample. Six primers were selected with the largest number of amplicons (Table 2).

Statistical analysis

Population genetic diversity

The profiles of amplified ISSR gels were recorded based on the presence (1) or absence (0) of fragments to construct the binary matrix used in the statistical analysis. The parameters of genetic diversity, such as percentage of polymorphic loci (*P*), the number of effective alleles (*Ne*), number of observed alleles (*Na*), Nei's genetic diversity (*He*), and Shannon index (*I*), were calculated using the POPGENE software v.1.32 (Yeh et al. 1997). Indirect gene flow (*Nm*) was calculated in POPGENE using the formula: $Nm = 0.5(1 - G_{ST})/G_{ST}$ (McDermott and McDonald 1993), where G_{ST} is the population differentiation of Nei's

Table 2 Optimized primers, sequences, and their respective annealing temperature, $MgCl_2$ concentration, and number of loci

Primer ISSR	Sequences (5′–3′)	Annealing (°C)	$MgCl_2$ (mM)	Loci
UBC 807 (AG)8-T	AGA GAG AGA GAG AGA GT	47	2.2	11
UBC 822 (TC)8-A	TCT CTC TCT CTC TCT CA	47	2.3	17
UBC826 (AC)8C	ACA CAC ACA CAC ACA CC	42	2.2	18
UBC 827 (AC)8-G	ACA CAC ACA CAC ACA CG	47	2.0	15
UBC 841 (GA)8-YC	GAG AGA GAG AGA GAG AYC	47	2.2	14
UBC 898 (CA)6-RY	CAC ACA CAC ACA RY	47	2.2	16
Total				96

R purine (A or G); Y pyrimidine (C or T)

coefficient (1987). The historic gene flow (*Nm*) was estimated between population pairs to determine those that contributed most to the movement of alleles.

Genetic structure

The analysis of molecular variance (AMOVA) and genetic differentiation (Φ_{ST}) was estimated using the ARLEQUIN v.3.5 software (Excoffier and Lischer 2010) to determine the structure of genetic variability of populations. We used 10,000 permutations to test for significance. The simplified representation of Nei's genetic identity (1987), obtained from POPGENE, was performed using a dendrogram construction based on Unweighted Pair-Group Method with Arithmetic mean (UPGMA).

The hypothesis of isolation by distance was tested using the Mantel correlation with the PC-program Ord 4.14 (McCune and Mefford 1999). For randomization of the data, we used the Monte Carlo method with 1000 random permutations for the significance matrix of correlations between population pairs of genetic distance (G_{ST}) and geographic distance (km). Principal component analysis (PCA) was performed using the program GenAlEx (Peakall and Smouse 2006).

Through the Structure v.2.3.4 software, we used a Bayesian analysis (Pritchard et al. 2000) to infer the number of genetic groups (K) that represent the sampled population. The number of genetic groups (K) ranged from $k = 1$ to $k = 15$, according to the Evanno et al. (2005), using the admixture ancestry model correlated with the frequency of alleles, and default parameters. Ten independent runs were performed for each K, using 500,000 simulations of Monte Carlo Markov Chain (MCMC) and a burn-in of 250,000. The number of populations K was identified according to the ΔK method (Evanno et al. 2005) as implemented in the Structure Harvester program (Earl and vonHoldt 2012).

Detection of recent population declines

We used the Bottleneck 1.2.02 software (Cornuet and Luikart 1997) to verify the occurrence of significant contemporary decreases in the effective size due to population reduction or recent colonization. We used the infinite allele model (IAM), based on Kimura and Crow (1964), and the stepwise mutation model (SMM) according to Kimura and Otha (1978). Luikart et al. (1998) suggest the use of SMM to analyze simple sequences (e.g., microsatellites) because it generates robust results. However, since the true mutation model

for most loci is intermediary between IAM and SMM, both models are recommended (Luikart et al. 1998). ISSRs and microsatellites are of similar origin (Godwin et al. 1997) and mutation should follow the same model. The sign test ($\alpha = 0.05$) was used from the frequency of alleles to reveal significant recent genetic bottlenecks (Luikart et al. 1998).

Results

ISSR markers

The six ISSR primers used in this study generated 91 polymorphic loci. The number of loci, as shown in Table 2, ranged between 11 and 18 per primer (mean = 15) with the sequence size ranging between 600 and 2500 bp. The final concentration of $MgCl_2$ was optimized for each primer and ranged from 2.0 to 2.3 mM (Table 2).

Population genetic diversity

The number of effective alleles (N_E) was equal to 1.571 (±0.261). Nei's genetic diversity (H_E) for the total population, assuming Hardy–Weinberg equilibrium, was 0.344 (±0.118) and the Shannon index (I) was 0.519 (±0.144). Populations CAR and PER (both from "Parque das Dunas") and BAR showed the highest genetic diversity index (H_E) (Table 3). The percentages of polymorphic loci in these populations were 96.7, 89.0, and 85.7%, respectively (Table 3).

Populations ESC and SAN in the state of Pernambuco showed the lowest percentage of polymorphic loci (P): 50.55 and 68.13%. However, ESC had higher genetic diversity index (H_E) than the populations GEN1, SAN, and PAB, which had the lowest H_E values (0.210, 0.232 and 0.260 respectively). These three populations also have a low percentage of polymorphic loci: 67.03, 68.13, and 78.02%, respectively.

The Shannon index (I) of genetic diversity for populations BAR (0.472) and CAR (0.468), in Rio Grande do Norte State, and MAT (0.439) in Paraíba, were higher than all other populations. On the other hand, GEN1 and SAN showed a lower Shannon index (0.323 and 0.366). Furthermore, the number of effective alleles (Ne) in GEN1 and SAN was also lower (1.345 and 1.370, respectively). Populations BAR, MAT, and NIV presented the highest number of effective alleles (Ne) (1.570, 1.511, and 1.496, respectively).

Among populations, Nei's genetic distance was lowest between PER and CAR (0.014) and between BAR and NIV (0.019) (Online Resource 1). We found the greatest genetic distance between ESC and PAB (0.503) and between ESC and PER (0.501). Nei's average genetic distance (G_{ST}) for all combinations was 0.177.

Genetic structure

For the analysis of molecular variance (AMOVA), we found a greater proportion of genetic variation between individuals within populations (60.85%) than between populations (Table 4). Of the total genetic diversity, 39.15% of the variation occurred between populations ($\Phi_{ST} = 0.391$, $P < 0.0001$). The Mantel test revealed the existence of a positive and significant correlation ($r = 0.794$; $P = 0.017$) between genetic and geographic distances between populations (Fig. 2).

Table 3 Estimates of genetic diversity in twelve populations of *Cattleya granulosa* in the Brazilian Atlantic forest

Populations	UC	*n*	*L*	*%P*	*Na*	*Ne*	H_E	*I*
ALC	−	10	74	81.32	1.813 ± 0.392	1.494 ± 0.362	0.287 ± 0.184	0.429 ± 0.252
BAR	+	10	78	85.71	1.857 ± 0.352	1.570 ± 0.372	0.321 ± 0.184	0.472 ± 0.247
NIV	+	10	73	80.22	1.802 ± 0.400	1.496 ± 0.372	0.287 ± 0.185	0.428 ± 0.254
MUR	−	20	72	79.12	1.791 ± 0.409	1.426 ± 0.353	0.255 ± 0.181	0.388 ± 0.251
PER	+	13	81	89.01	1.890 ± 0.314	1.401 ± 0.293	0.256 ± 0.152	0.401 ± 0.207
CAR	+	22	88	96.70	1.967 ± 0.179	1.499 ± 0.300	0.305 ± 0.141	0.468 ± 0.181
GEN1	+	12	61	67.03	1.670 ± 0.473	1.345 ± 0.347	0.210 ± 0.184	0.323 ± 0.263
GEN2	+	13	70	76.92	1.769 ± 0.424	1.471 ± 0.346	0.279 ± 0.180	0.417 ± 0.255
PAB	−	14	73	80.22	1.802 ± 0.4005	1.422 ± 0.321	0.260 ± 0.169	0.398 ± 0.237
MAT	−	11	71	78.02	1.780 ± 0.416	1.511 ± 0.351	0.297 ± 0.183	0.439 ± 0.257
ESC	−	6	46	50.55	1.780 ± 0.418	1.489 ± 0.382	0.281 ± 0.191	0.419 ± 0.262
SAN	−	10	62	68.13	1.838 ± 0.371	1.370 ± 0.323	0.232 ± 0.164	0.366 ± 0.225

Populations in protected areas (UC), sample size (*n*), polymorphic loci (*L*), percentage of polymorphic loci (*%P*), number of alleles (*Na*), effective number of alleles (*Ne*), genetic diversity (H_E), Shannon index of genetic diversity (*I*). The values shown are mean ± standard deviation

The historic gene flow (*Nm*), obtained by indirect calculation between pairs of populations (McDermott and McDonald 1993), was highest between PER and CAR (35.21), VIN and BAR (24.50), and between MAT and PAB (14.65). The lowest values were observed between the ESC population (Pernambuco) and all others, with an average of 0.6 migrants. The average historical gene flow among all populations was 2.4.

Based on the genetic identity values, the dendrogram (Fig. 3) suggests the existence of two groups, separating populations ESC and SAN (cluster 1) from the other populations (cluster 2). Cluster (1) has a greater geographical distance from the others, as well as a higher genetic distance and lower H_E value when compared with other groups or populations. PCA revealed that the first and third major component explained most of the total variation (PC 1 = 53.22% and PC 3 = 10.78%) (Online Resource 2).

The Bayesian analysis revealed the existence of five distinct genetic groups (k = 5) (Online Resource 3). The value of K is the actual number of genetic groups, based on the ΔK method. Thus, the 12 sampled populations are structured into five groups (Fig. 4) group I comprises MUR population; group II included populations GEN1 + GEN2 + -PER + CAR (also at both k = 3 and k = 4); group III with NIV + BAR + ALC populations; group IV represented by MAT + PAB populations; and group V with SAN + ESC populations.

Table 4 Molecular analysis of variance (AMOVA) in populations of *Cattleya granulosa*

Source of variation	*df*	SS	Variance components	Total variance (%)	*P*
Among populations	11	2.645	0.01736	39.15	<0.0001
Within populations	137	3.697	0.02699	60.85	<0.0001
Total	148	6.342	0.04434	100	
Φ_{ST}	0.39146				

DF degrees of freedom, *SS* sum of squared deviations, Φ_{ST} genetic differentiation

Detection of recent population declines

Based on tests of IAM and SMM models, only four populations (ALC, MUR, GEN2, and MAT) showed balance between mutation and drift. For the other eight populations (BAR, NIV, PER CAR, GEN1, PAB, ESC, and SAN), the models strongly indicate the occurrence of recent population bottlenecks (Table 5). For ESC and SAN, the signal test revealed a highly significant heterozygosity deficit ($P < 0.0001$) for both IAM and SMM models.

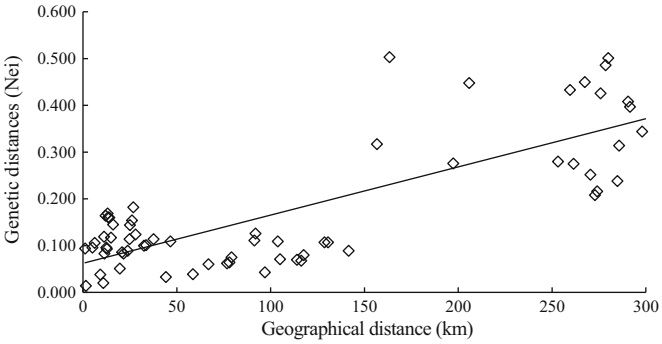

Fig. 2 Relationship between genetic and geographic distances between populations of *C. granulosa*

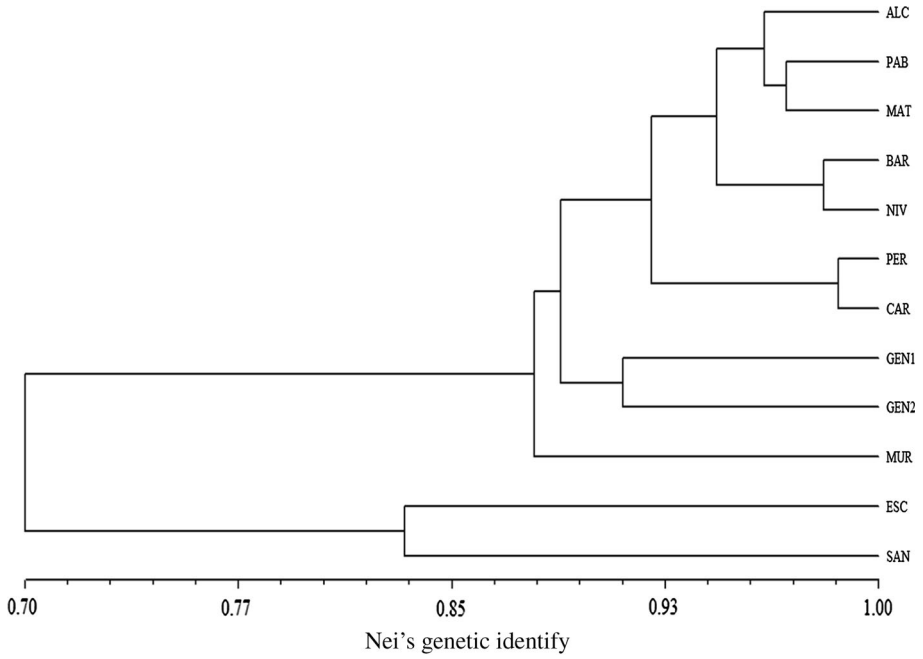

Fig. 3 Cluster analysis (UPGMA) of Nei's genetic identity among populations of *C. granulosa*

Discussion

Population genetic diversity

The use of ISSR markers demonstrates the existence of high genetic polymorphism in populations of *Cattleya granulosa*. Large percentages of polymorphic loci are considered common in some species in the *Cattleya* genus. In fact, this condition was observed in eight species of the genus using molecular markers (Benner et al. 1995), as well as in *C. labiate*, analyzed using both ISSR and RAPD markers (Pinheiro et al. 2012).

For *C. granulosa*, more than half of the studied populations present a percentage of polymorphic loci greater than 70% (ALC, BAR, NIV, MUR, PER CAR, GEN2, PAB, MAT); the lowest value (50.55%) was found for the ESC population. This lower number of polymorphic loci is probably due to the limited number of individuals found in this population (only six individuals were sampled), and low population density. Using dominant ISSR markers, Qian et al. (2013) found similar results for the rare and endemic *Calanthe tsoongiana* orchid, with 96.8% polymorphic loci, but half of the population exhibited polymorphism below 50%.

The number of loci used in genetic diversity studies of orchid species using ISSR molecular markers varies considerably: for *Octomeria crassifolia* and *O. grandiflora*, 92 and 98 loci were evaluated, respectively (Barbosa et al. 2013); for *Calanthe tsoongiana*, 124 loci were used (Qian et al. 2013). The ratio between monomorphic and polymorphic

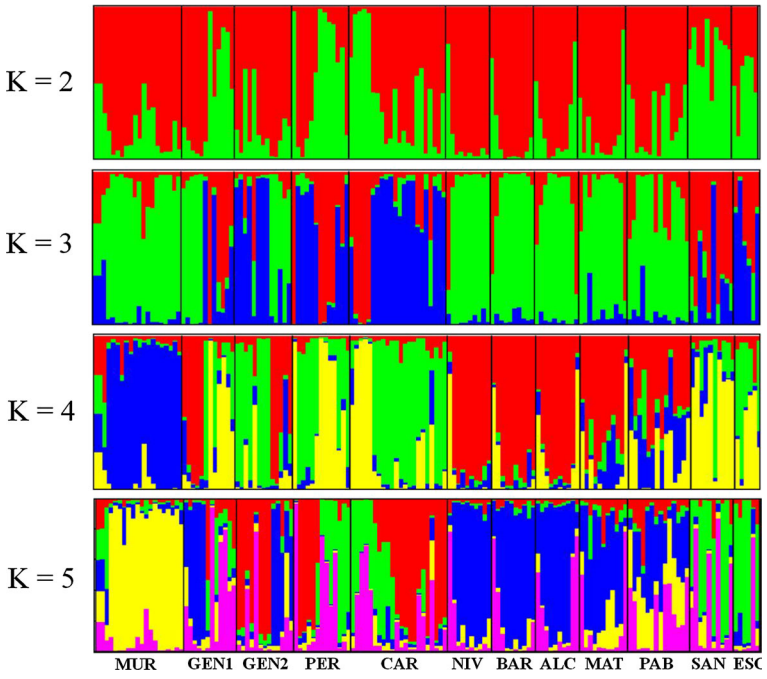

Fig. 4 Bayesian analysis of *Cattleya granulosa* with their proportion of genotypes in the sampled populations. Number of clusters (K) ranging from 2 to 5 are shown. Populations are bounded by *black vertical bar*

Table 5 Bottleneck tests for the populations of *Cattleya granulosa* using IAM and SMM models

IAM				SMM		
Populations	n	H*d*/H*e*	*P*	n	H*d*/H*e*	*P*
ALC	51	46/45	0.13897	51	48/43	0.05797
BAR	53	35/56	0.27065	42	35/56	0.00318**
NIV	53	47/44	0.04012*	42	47/44	0.41654
MUR	43	46/45	0.40850	47	49/42	0.14464
PER	39	55/36	0.26576	49	58/33	0.00048**
CAR	39	34/57	0.00008**	48	42/49	0.44775
GEN1	36	63/28	0.04555*	45	68/23	0.00000**
GEN2	37	49/42	0.16583	42	53/38	0.24080
PAB	42	46/45	0.28812	52	52/39	0.00393**
MAT	47	39/52	0.19407	46	41/50	0.25445
ESC	48	65/26	0.00000**	47	65/26	0.00001**
SAN	48	70/21	0.00000**	47	70/21	0.00000**

n, expected number of loci with excess heterozygosity under the respective model; Hd/He, number of loci with deficit of heterozygosity and excess heterozygosity; *P,* probability

*, ** Significant at the 5 and 1% probability, respectively

loci is an important parameter that can be obtained from dominant markers and used in genetics studies of conserved populations (Cota et al. 2011; Oliveira et al. 2012).

The distribution of genetic variability within and between populations can be influenced by the life history and reproductive system, both of which have significant impacts on a species (Nybom and Bartish 2000). For example, epiphytic orchids have higher diversity indices compared to terrestrial orchids because they have different strategies to survive; examples include the occurrence of allogamous and autogamous individuals, and perennials with long life expectancy (Ávila-Diaz and Oyama 2007). In fact, the genetic diversity indices for *C. granulosa* ($H_E = 0.344$; $I = 0.519$) were very similar to those found for other epiphytic orchids, such as *Octomeria crassifolia* ($H_E = 0.352$; $I = 0.530$) and *O. grandiflora* ($H_E = 0.338$; $I = 0.508$) (Barbosa et al. 2013), and slightly lower than the results found for *Calanthe tsoongiana* ($H_E = 0.398$; $I = 0.576$) (Qian et al. 2013). However, when compared to threatened and endemic terrestrial orchids, the genetic diversity index of *C. granulosa* was greater than *Piperia yadonii* R. Morgan and Ackerman ($H_E = 0.06$; George et al. 2009), *Paphiopedilum micranthum* Tang and Wang ($H_E = 0.15$; Li et al. 2002), and *Changnienia amoena* SS Chien ($H_E = 0.12$; Li and Ge 2006). In comparison with closely phylogenetically related species, it appears that the genetic diversity of *C. elongata* ($H_E = 0.175$) (Cruz et al. 2011) is lower than *C. granulosa.* Similarly, *C. labiata* ($H_E = 0.17$; $I = 0.25$), as well as other species of the same genus (*C. gaskelliana*, *C. lawrenceana*, *C. wallissi*, *C. warneri*, *C. warscewiczii*, *C. lueddemanniana*, *C. maxima*, *C. percivaliana*, *C. schroeder*, *C. trianae*; $H_E = 0.22$; $I = 0.36$) also showed lower genetic diversity (Pinheiro et al. 2012).

The expected average genetic differentiation (G_{ST}) in Orchidaceae has been estimated at 0.087 (Hamrick and Godt 1996). Using this parameter, the level of genetic differentiation among populations of *C. granulosa* ($G_{ST} = 0.177$) is higher than expected for the family. However, population patterns obtained for the family have shown heterogeneity, with G_{ST} values ranging from 0.012 in *Pterostylis angusta* A.S. George, an endemic species (Sharmaa et al. 2001), and 0.924 for *Zeuxine strateumatica* (Ln.) Schltr., a terrestrial orchid with restricted distribution (Sun and Wong 2001). In comparison, the average G_{ST} value for *C. granulosa* is similar to that estimated for perennial and long life species ($G_{ST} = 0.19$), species with a mixed breeding system ($G_{ST} = 0.20$), anemochoric species ($G_{ST} = 0.17$),

and endemic species ($G_{ST} = 0.18$) (Nybom 2004). This supports the hypothesis that the majority of these species and their remaining populations still retain genetic variability, an important condition for their survival and maintenance over time.

Geographic patterns of genetic variation

The AMOVA revealed high genetic differentiation between populations of *C. granulosa* (39.15%). This level of differentiation is greater than that observed among populations of *C. elongata* (18%; Cruz et al. 2011) and *C. labiata* (15%; Pinheiro et al. 2012), both endemic to northeastern Brazil. However, the differentiation value was similar to the value described for mixed reproduction species ($\Phi_{ST} = 40\%$) (Nybom 2004). This is consistent with the reproductive system of *C. granulosa* which is self-compatible and dependent on pollinators for sexual reproduction (Costa 2010).

Natural selection alters gene frequencies and works alongside migration, dispersion, and other processes that affect genetic structure, such as isolation by distance (Wright 1951). This is the main model used to explain how subdivisions can occur in large, continuous populations that are randomly distributed. In this respect, the high genetic differentiation of *C. granulosa* provides evidence of the spatial segregation of genotypes, as expected. A positive and significant correlation between genetic distance and geographic distance of the *C. granulosa* populations ($r = 0.794$, $P = 0.017$) was similar to that found for *C. labiata* ($r = 0.56$; $P = 0.0001$; Pinheiro et al. 2012). However, this pattern is in contrast with that seen for *C. elongata* ($r = -0.0742$, $P = 0.4924$; Cruz et al. 2011), which presented no isolation by distance despite having discontinuous populations and being located in areas where spatial genetic segregation in other plant species have been reported (Cruz et al. 2011).

An important factor that should be considered in the genetic structure of *C. granulosa* is the significant rate of fragmentation of the Atlantic Forest biome. In recent decades, the northeastern region of Brazil has experienced extensive urban and agricultural expansion. Because of this fragmentation, *C. granulosa* populations generally occur in isolated Urban Parks, in the remaining natural vegetation adjacent to large municipalities, and in the vegetation surrounding sugarcane monocultures and mines. Large areas of cultivated sugarcane in the states of Paraíba and Pernambuco exacerbate the isolation of *C. granulosa* populations and its habitat.

Forest fragmentation, or even the loss of natural habitats, can lead to an erosion of genetic variability related to changes in plant breeding systems. This results in inbreeding and reductions to the rate of gene flow, which in turn lead to increased differentiation between populations (Young et al. 1996; Jump and Penuelas 2006). The reproductive isolation of populations, together with demographic decline, can cause a loss of alleles due to genetic drift if gene flow is not restored through subsequent generations (Couvet 2002).

The dendrogram (UPGMA) and PCA approaches used in this study to differentiate the genotype groupings of *C. granulosa* showed similar results in the genetic structure of the populations. From genetic identity, the groupings were among geographically proximal populations. For example, the ESC-SAN cluster consists of two populations occurring at the southern range of the sample, that are geographically contiguous (26.8 km), but with an average distance of 263.8 km from other populations. These results are consistent with the groups generated through PCA on the basis of Nei's genetic distance. Through the Bayesian analysis, we were able to identify geographic patterns of intra-group distribution of genotypes, indicating the existence of five distinct genetic groups ($K = 5$). Some sampled populations have lower genetic structure due to sharing of the same genotypes in

similar proportions, such as NIV-BAR-ALC (group III), MAT-PAB (group IV), and SAN-ESC (group V). These populations have alleles with higher frequency, but not exclusive alleles, indicating a historic connection between them, except the MUR population (group I) that had a higher frequency of a single genotype. The group II showed different proportions of genotypes, which results in their classification as genetically distinct populations and consequently distinct units for genetic conservation.

Gene flow (*Nm*) is crucial to the homogenization of alleles between populations (Hartl and Clark 2007). Despite the significant geographical distance between some populations of *C. granulosa* (average of 111.2 km), historical gene flow occurred between them, although the average *Nm* across all populations was low ($Nm = 2.4$). Geographically, the genetic differentiation among the sampled populations is lower in the central region of its geographical distribution, represented by combinations of populations ALC, MAT, and PAB, that are phylogenetically similar and likely contributed to the dynamics of historic gene flow between populations.

Recent population bottlenecks

Populations that are balanced between mutation and genetic drift have equal probability that a locus presents an excess or deficit of heterozygotes, assuming the population size has remained constant in the recent past (Cornuet and Luikart 1997). On the other hand, recently reduced populations that underwent a bottleneck exhibit a rapid reduction in the number of alleles and a temporary excess of heterozygosity (Luikart et al. 1998; Cornuet and Luikart 1997). This condition is present in the populations of *C. granulosa* in Pernambuco (ESC and SAN), considering the IAM and SMM models ($\alpha = 0.01$).

The detection of populations that have experienced recent population bottlenecks have obvious implications for the species' evolution and conservation, especially considering the risk of local extinction as a result of reduced population size (Luikart et al. 1998; Lee et al. 2002). The identification of population bottlenecks enables us to identify populations that are critical for conservation. Thus, the detected recent genetic bottleneck, the historically low gene flow between the two Pernambuco populations and the other studied populations ($Nm = 0.49–1.90$), as well as the irreversible situation of *C. granulosa* population fragmentation, demonstrates the urgent need for conservation of the remaining populations of the region.

Implications for genetic conservation

Indices of genetic diversity and structure estimated from the allele frequencies are important to support conservation measures and to identify populations that are a priority for conservation. However, management practices must be combined with the demographic situation of the populations. It is clear that *C. granulosa* has undergone a drastic population decline resulting from the high degree of fragmentation of the Atlantic Forest in northeastern Brazil, loss of habitats due to human occupation, and predation. In this biome, *C. granulosa* occurs in the states of Rio Grande do Norte, Paraíba, Bahia (Martinelli and Moraes 2013), and Pernambuco. Although the levels of genetic diversity found in this study highlights the ecological, in disturbed environments a loss of genetic variability and fitness of the species may occur. Increases in the amount of land being used for agriculture in Pernambuco and Paraíba is the main driver responsible for the disappearance of the *C. granulosa* habitat. In the short term, this situation increases the risk of local population

extinction, and in the long term it limits the evolutionary potential of the species to respond to climatic and environmental changes (Young et al. 1996; Aguilar et al. 2008).

Although some populations are officially protected in conservation areas, such as the "Parque Estadual das Dunas", "APA de Genipabu", "Parque Dom Nivaldo", and "Barreira do Inferno", other areas continue to suffer the dramatic consequences of the illegal removal of specimens. Despite efforts to curb poaching of the species, they are still being removed, even from protected areas.

Like other species of orchids (Li and Ge 2006), *C. granulosa* generally have a habitat preference and they are dependent on pollinators, although also self-compatible. The reproductive biology of *C. granulosa* shows that the reproductive success of the species depends on visits from pollinators, which are rare, thus resulting in low fruit production (Costa 2010). Therefore, due to the easy access to the plants, which are generally located in local and accessible forest habitats, artificial pollination, although laborious, could be a viable alternative for facilitating genetic variability within *C. granulosa* populations. The genetic data presented herein provides important information that can be applied to the conservation of the species, informing strategies for the management of natural populations. However, we emphasize that the artificial transplant of individuals among different populations or regions, as has been proposed for endangered species (Hamrick and Godt 1996; Kingston et al. 2004), should be practiced with caution. Clearly, there is a need for future studies that assess the geographical distribution of pollinators and their behavior in certain habitats of *C. granulosa* (Costa 2010). In addition, landscape management strategies should consider both the creation of vegetation corridors and the protection of forest remnants, considering the practically irreversible fragmentation of populations (Vieira and Carvalho 2008; Brandão et al. 2015).

Finally, in light of the genetic data gathered in this study, it is clear that the total genetic diversity of the species should be conserved and more populations should be protected to maximize species conservation across its entire geographic distribution. This is particularly applicable for those populations with different genetic compositions or with high genetic diversity (e.g., ESC, SAN, PAB, MAT, ALC, MUR) that must be prioritized through ex situ or in situ conservation strategies.

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References

Aguilar R, Quesada M, Ashworth L, Herrerias-Diego Y, Lobo J (2008) Genetic consequences of habitat fragmentation in plant populations: susceptible signals in plant traits and methodological approaches. Mol Ecol 17:5177–5188

Ávila-Diaz I, Oyama K (2007) Conservation genetics of an endemic and endangered epiphytic *Laelia speciosa* (Orchidaceae). Am J Bot 94(2):184–193

Barbosa AR, Silva-Pereira V, Borba EL (2013) High genetic variability in self-incompatible myophilous *Octomeria* (Orchidaceae, Pleurothallidinae) species. Braz J Bot 36(3):179–187

Benner MS, Braunstein MD, Weisberg MU (1995) Detection of DNA polymorphisms within the genus *Cattleya* (Orchidaceae). Plant Mol Biol Rep 13:147–155

Borba EL, Braga PIS (2003) Reproductive biology of *Pseudolaelia corcovadensis* Porto & Brade (Orchidaceae): melittophyly and selfcompatibility in a basal Laeliinae. Braz J Bot 26(4):541–549

Borba EL, Felix JM, Solferini VN, Semir J (2001) Fly-pollinated *Pleurothallis* (Orchidaceae) species have high genetic variability: evidence from isozyme markers. Am J Bot 88:419–428

Borba EL, Funch RR, Ribeiro PL, Smidt EC, Silva-Pereira V (2007) Demography, and genetic and morphological variability of the endangered *Sophronitis sincorana* (Orchidaceae) in the Chapada Diamantina, Brazil. Plant Syst Evol 267:129–146

Brandão MM, Vieira FA, Nazareno AG, Carvalho D (2015) Genetic diversity of neotropical tree *Myrcia splendens* (Myrtaceae) in a fragment-corridor system in the Atlantic rainforest. Flora 216:35–41

Camara-Neto C, Chaves-Camara I, Medeiros SC, Braga MRA (2007) Rescuing *Cattleya granulosa* Lindley in the wild. Lankesteriana 7(2):243–246

Case MA, Mlodozeniec HT, Wallace LE, Weldy TW (1998) Conservation genetics and taxonomic status of the rare Kentucky lady's slipper: *cypripedium kentuckiense* (Orchidaceae). Am J Bot 85:1779–1786

Chase MW, Cameron KM, Barrett R, Freudenstein JV (2003) DNA data and Orchidaceae systematics: a new phylogenic classification. In: Dixon KW, Kell SP, Barrett RL, Cribb PJ (eds) Orchid conservation. Natural History, Kota Kinabalum, pp 69–90

Chung MY (2009) Lack of allozyme diversity in populations of the rare, endangered terrestrial orchids *Tipularia japonica* and *Epipactis papillosa* in Korea. Plant Syst Evol 278:203–209

Cornuet JM, Luikart G (1997) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. Genetics 144:2001–2014

Costa RA (2010) Biologia floral e sistema reprodutivo de *Cattleya granulosa* Lindl., uma Orchidaceae ameaçada e endêmica do Nordeste do Brasil, p 62. Dissertation—Master Degree in Vegetal Biology, Universidade Federal de Pernambuco, Recife, PE

Cota LG, Vieira FA, Melo-Júnior AF, Brandão MM, Santana KNO, Guedes ML, Oliveira DA (2011) Genetic diversity of *Annona crassiflora* (Annonaceae) in northern Minas Gerais State. Genet Mol Res 10:2172–2180

Couvet D (2002) Deleterious effects of restricted gene flow in fragmented populations. Conserv Biol 16:369–376

Cozzolino S, Cafasso D, Pellegrino G, Musacchio A, Widmer A (2003) Fine-scale phylogeographical analysis of Mediterranean *Anacamptis palustris* (Orchidaceae) populations based on chloroplast minisatellite and microsatellite variation. Mol Ecol 12:2783–2792

Cruz DT, Borba EL, van den Berg C (2003) O gênero *Cattleya* no estado da Bahia. Sitientibus 3(1–2):28–36

Cruz DT, Selbah-Schnadelbach A, Lambert SM, Ribeiro PL, Borba EL (2011) Genetic and morphological variability in *Cattleya elongata* Barb. Rodr. (Orchidaceae), endemic to the campo rupestre vegetation in northeastern Brazil. Plant Syst Evol 294:87–98

Doyle JJ, Doyle JL (1987) A rapid DNA isolation method for small quantities of fresh tissues. Phytochem Bull Bot Soc Am 19:11–15

Earl DA, vonHoldt BM (2012) Structure Harvester: a website and program for visualizing Structure output and implementing the Evanno method. Conserv Genet Resour 4(2):359e361

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol 14:2611–2620

Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Resour 10:564–567

Fajardo CG, Vieira FA, Molina WF (2014) Interspecific genetic analysis of orchids in Brazil using molecular markers. Plant Syst Evol 300:1825–1832

George S, Sharma J, Yadon VL (2009) Genetic diversity of the endangered and narrow endemic *Piperia Yadonii* (Orchidaceae) assessed with ISSR polymorphisms. Am J Bot 96(11):2022–2030

Godwin ID, Aitken EAB, Smith LW (1997) Application of inter simple sequence repeat (ISSR) markers to plant genetics. Electrophoresis 18:1524–1528

Gustafsson S (2000) Patterns of genetic variation in *Gymnadenia conopsea*, the fragrant orchid. Mol Ecol 9:1863–1872

Hamrick JL, Godt MJW (1996) Effects of life history traits on genetic diversity in plant species. Philos Trans R Soc Lond B Biol Sci 351:1291–1298

Hartl DL, Clark AG (2007) Principles of population genetics. Sinauer Associates, Sunderland

Jump AS, Penuelas J (2006) Genetic effects of chronic habitat fragmentation in a wind-pollinated tree. Proc Natl Acad Sci USA 103(21):8096–8100

Kimura M, Crow J (1964) The number of alleles that can be maintained in a finite population. Genetics 49:725–738

Kimura M, Otha T (1978) Stepwise mutation model and distribution of allelic frequencies in a finite populations. Proc Natl Acad Sci USA 75:2868–2872

Kingston N, Waldren S, Smyth N (2004) Conservation genetics and ecology of *Angiopteris chauliodonta* Copel. (Marattiaceae), a critically endangered fern from pitcairn Island, South Central Pacific Ocean. Biol Conserv 117:309–319

Lee S-L, Ng KKS, Saw L-G, Norwati A, Salwana MHS, Lee C-T, Norwati M (2002) Population genetics of *Intsia palembanica* (Leguminosae) and genetic conservation of virgin jungle reserves in Peninsular Malaysia. Am J Bot 89(3):447–459

Li A, Ge S (2006) Genetic variation and conservation of *Changnienia amoena*, an endangered orchid endemic to China. Plant Syst Evol 258:251–260

Li A, Luo Y, Ge S (2002) Premilary study on conservation genetics of an endangered orchid (*Paphiopedilum micranthum*) from southwestern China. Biochem Genet 40:195–201

Loveless MD, Hamrick JL (1984) Ecological determinants of genetic-structure in plant-populations. Annu Rev Ecol Evol Syst 15:65–95

Luikart G, Allendorf FW, Cornuet JM, Sherwin WB (1998) Distortion of allele frequency distributions provides a test for recent population bottlenecks. J Hered 89(3):238–247

Martinelli G, Moraes MA (2013) Livro vermelho da flora do Brasil. Instituto de Pesquisas Jardim Botânico do Rio de Janeiro, Rio de Janeiro

McCune B, Mefford MJ (1999) Multivariate analysis of ecological data. Gleneden Beach, Oregon

McDermott JM, McDonald BA (1993) Gene flow in plant pathosystems. Annu Rev Phytopathol 31:353–373

Ministério do Meio Ambiente (2008) Instrução normativa n° 6 de 23 de setembro de 2008. http://www.mma.gov.br/estruturas/179/_arquivos/179_05122008033615.pdf. Accessed 07 July 2016

Nei M (1987) Molecular evolutionary genetics. Columbia University Press, New York

Niknejad A, Kadir MA, Kadzimin SB, Abdullah NAP, Sorkheh K (2009) Molecular characterization and phylogenetic relationships among and within species of *Phalaenopsis* (Epidendroideae: Orchidacheae) base on RAPD analysis. Afr J Biotechnol 8:5225–5240

Nybom H (2004) Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. Mol Ecol 13:1143–1155

Nybom H, Bartish IV (2000) Effects of life history traits and sampling strategies on genetic diversity estimates obtained with RAPD markers in plants. Perspect Plant Ecol Evol Syst 3(2):93–114

Oliveira DA, Melo-Júnior AF, Brandão MM, Rodrigues LA, Menezes EV, Ferreira PRB (2012) Genetic diversity in populations of *Acrocomia aculeata* (Arecaceae) in the northern region of Minas Gerais, Brazil. Genet Mol Res 11(1):531–538

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

Parab GV, Krishnan S (2008) Assessment of genetic variation among populations of *Rhynchostylis retusa*, an epiphytic orchid from Goa, India using ISSR and RAPD markers. Indian J Biotechnol 7:313–319

Parab GV, Krishnan S, Janarthanam MK, Sivaprakash KR, Parida A (2008) ISSR and RAPD markers assessed genetic variation of *Aerides maculosum*—an epiphytic orchid from Goa, India. J Plant Biochem Biotechnol 17(1):107–109

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes 6:288–295

Pinheiro LR, Rabbani ARC, Silva AVC, Lédo AS, Pereira KLG, Diniz LEC (2012) Genetic diversity and population structure in the Brazilian *Cattleya labiata* (Orchidaceae) using RAPD and ISSR markers. Plant Syst Evol 298:1–11

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959

Qian X, Wang C-X, Tian M (2013) Genetic diversity and population differentiation of *Calanthe tsoongiana*, a rare and endemic orchid in China. Int J Mol Sci 14(10):20399–20413

Rodrigues JF, van den Berg C, Abreu AG, Novello M, Veasey EA, Oliveira GCX, Koehler S (2015) Species delimitation of *Cattleya coccinea* and *C. mantiqueirae* (Orchidaceae): insights from phylogenetic and population genetics analyses. Plant Syst Evol 301:1345–1359

Sharmaa IK, Jonesa DL, Younga AG, French CJ (2001) Genetic diversity and phylogenetic relatedness among six endemic *Pterostylis* species (Orchidaceae; series Grandiflorae) of Western Australia, as revealed by allozyme polymorphisms. Biochem Syst Ecol 29:697–710

Silva-Pereira V, Smidt EC, Borba EL (2007) Isolation mechanisms between two sympatric *Sophronitis* (Orchidaceae) species endemic to Northeastern Brazil. Plant Syst Evol 269(3–4):171–182

Soto Arenas MA, Solano Gómez R, Hágsater E (2007) Risk of extinction and patterns of diversity loss in Mexican orchids. Lankesteriana 7:114–121

Sun M, Wong KC (2001) Genetic structure of three orchid species with contrasting breeding systems using RAPD and allozyme markers. Am J Bot 88:2180–2188

Swarts ND, Dixon KW (2009) Terrestrial orchid conservation in the age of extinction. Ann Bot 104:543–556

Toro MA, Caballero A (2005) Characterization and conservation of genetic diversity in subdivided populations. Philos Trans R Soc Lond B Biol Sci 360(1459):1367–1378

Trapnell DW, Hamrick JL (2005) Mating patterns and gene flow in the neotropical epiphytic orchid, *Laelia rubescens*. Mol Ecol 14:75–84

Trapnell DW, Hamrick JL, Nason JD (2004) Three-dimensional fine-scale genetic structure of the neotropical epiphytic orchid, *Laelia rubescens*. Mol Ecol 13:1111–1118

Trapnell DW, Hamrick JL, Ishibashi CD, Kartzinel TR (2013) Genetic inference of epiphytic orchid colonization; it may only take one. Mol Ecol 22:3680–3692

Ueno S, Rodrigues JF, Alves-PereiraA Pansarin ER, Veasey EA (2015) Genetic variability within and among populations of an invasive, exotic orchid. AoB Plants 7:plv077. doi:10.1093/aobpla/plv077

Vale Á, Rojas D, Álvarez JC, Navarro L (2013) Distribution, habitat disturbance and pollination of the endangered orchid *Broughtonia cubensis* (Epidendrae: Laeliinae). Bot J Linn Soc 172(3):345–357

Van den Berg C (2014) Reaching a compromise between conflicting nuclear and plastid phylogenetic trees: a new classification for the genus *Cattleya* (Epidendreae; Epidendroideae; Orchidaceae). Phytotaxa 186(2):075–086

Van den Berg C, Higgins W, Dressler R, Whitten W, Soto-Arenas M, Chase MA (2009) Phylogenetic study of Laeliinae (Orchidaceae) based on combined nuclear and plastid DNA sequences. Ann Bot 104:417–430

Velozo HP, Rangel-Filho ALR, Lima JCA (1991) Classificação da vegetação brasileira adaptada a um sistema universal. IBGE, Rio de Janeiro

Vieira FA, Carvalho D (2008) Genetic structure of an insect-pollinated and bird-dispersed tropical tree in vegetation fragments and corridors: implications for conservation. Biodivers Conserv 17:2305–2321

Wallace LE (2003) Molecular evidence for allopolyploid speciation and recurrent origins in *Platanthera huronensis* (Orchidaceae). Int J Plant Sci 164:907–916

Wallace LE (2004) A comparison of genetic variation and structure in the allopolyploid *Platanthera huronensis* and its diploid progenitors, *Platanthera aquilonis* and *Platanthera dilatata* (Orchidaceae). Can J Bot 82:244–252

Wright S (1951) The genetical structure of populations. Ann Eugen 15(4):313–354

Yeh FC, Yang RC, Boyle TBJ, Ye ZH, Mao JX (1997) POPGENE, the user-friendly shareware for population genetic analysis. Molecular Biology and Biotechnology Center, University of Alberta, Edmonton

Young A, Boyle T, Brown T (1996) The population genetic consequences of habitat fragmentation for plants. Trends Ecol Evol 11(10):413–418