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PHYLOGEOGRAPHIC STRUCTURE AND OUTBREEDING DEPRESSION REVEAL EARLY STAGES OF REPRODUCTIVE ISOLATION IN THE NEOTROPICAL ORCHID *EPIDENDRUM DENTICULATUM*

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Phylogeographic studies provide an important framework for investigating the mechanisms operating during the earliest stages of speciation, as reproductive barriers can be examined among divergent lineages in a geographic context. We investigated the evolution of early stages of intrinsic postmating isolation among different populations and lineages of *Epidendrum denticulatum*, a Neotropical orchid distributed across different biomes in South America. We estimated genetic diversity and structure for both nuclear and plastid markers, using a haplotype network, differentiation tests, Bayesian assignment analysis, and divergence time estimates of the main lineages. Reproductive barriers among divergent lineages were examined by analyzing seed viability following reciprocal crossing experiments. Strong plastid phylogeographic structure was found, indicating that *E. denticulatum* was restricted to multiple refuges during South American forest expansion events. In contrast, significant phylogeographic structure was not found for nuclear markers, suggesting higher gene flow by pollen than by seeds. Large asymmetries in seed set were observed among different plastid genetic groups, suggesting the presence of polymorphic genic incompatibilities associated with cytonuclear interactions. Our results confirm the importance of phylogeographic studies associated with reproductive isolation experiments and suggest an important role for outbreeding depression during the early stages of lineage diversification.

KEY WORDS: **Genetic structure, microsatellites, multiple refuges, Orchidaceae, phylogeography, reproductive barriers, South America.**

Phylogeographic studies have provided a basic and unifying framework for understanding speciation mechanisms in a geographic context (Hewitt 2001; Avise 2009). Past climatic shifts have had a dramatic impact on the geographic distribution of organisms, leading to limited gene exchange among fragmented populations (Avise 2009). The effects of such population range fluctuations on species and lineage diversification offer an interesting opportunity to investigate microevolutionary processes

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associated with speciation events (Diniz-Filho et al. 2008; Avise 2009). The role of historically climate-stable regions or refuges in the diversification of lineages and species has been confirmed (i.e., Bennett and Provan 2008; Avise 2009). For instance, multiple refuges have been identified in most phylogeographic studies performed in South America (reviewed by Turchetto-Zolet et al. 2013), specially for those organisms occurring in different biomes (Novaes et al. 2010; Pinheiro et al. 2011). Maps of historically stable areas have been generated by climate-based modeling for different Neotropical biomes (Brazilian Atlantic Forest, Carnaval & Moritz 2008; Cerrado, Werneck et al. 2012a) and have contributed to testing hypothesis concerning genetic diversity levels and demographic scenarios in temporally stable (refuges) and unstable (recently colonized) regions (Carnaval et al. 2009; Thomé et al. 2010; Martins 2011; Amaro et al. 2012; Werneck et al. 2012b). In general, it is expected that populations sampled at climate stable regions show higher levels of genetic diversity than populations sampled at recently colonized regions due to founder effects (Hewitt 2001; Bennett and Provan 2008). However, some studies have not corroborated this expectation (e.g., Thomé et al. 2010; Werneck et al. 2012b).

A particularly interesting topic in speciation research concerns the interaction between the geographic differentiation of lineages and the evolution of reproductive isolation (RI) within species (Hewitt 2001; Avise et al. 2009). A detailed picture of phylogeographic patterns observed within species is crucial to reach this goal (Lee 2000; Gómez et al. 2007; Skrede et al. 2008). According to the biological species concept (Mayr 1942), speciation can be viewed as the evolution of RI between formerly interbreeding populations (Coyne and Orr 2004). The identification of reproductive barriers between different species does not essentially correspond to the mechanisms that drive divergence during early stages of speciation, as isolation barriers could arise after speciation, by divergent selection and genetic drift (Coyne and Orr 2004; Scopece et al. 2010). Therefore, an investigation of reproductive barriers within species can provide insight into the processes operating during the earliest stages of speciation, as the origin and fixation of genetic characters are properties of population-level processes (Scopece et al. 2010).

Reproductive barriers are often classified as either pre- or postmating in sexually reproducing organisms such as most flowering plants (Coyne and Orr 2004). When premating barriers are weak, due to coincident phenology or pollinator sharing, strong postmating pre- and postzygotic barriers prevent gene flow across species (Scopece et al. 2007; Lowry et al. 2008). An extensive number of pre- and postzygotic barriers have been identified within species (reviewed by Scopece et al. 2010), which are frequently attributed to incompatibilities involving cytonuclear or gametophyte–sporophyte interactions based in the Bateson–Dobzhansky–Muller (BDM) classic model of genic incompatibilities (Turelli and Moyle 2007). According to this model, negative epistasis is observed among nuclear and organellar alleles that have followed independent evolutionary trajectories after divergence of a lineage. BDM incompatibilities are the major genetic source of hybrid inviability and sterility (Coyne and Orr 2004) and the negative effects of nuclear–organellar interactions can be an important intrinsic postmating reproductive barrier in plants (Martin and Willis 2010; Greiner et al. 2011; Palma-Silva et al. 2011; Zitari et al. 2012). Such genetic incompatibilities are expected to be polymorphic within species, as alleles are still not fixed among recently divergent lineages (Bomblies and Weigel 2007; Scopece et al. 2010; Cutter 2012). Recent studies have supported the expectation that the initial stages of isolation, and their underlying genetics, can be complex (Lexer and Widmer 2008; Bomblies 2009, 2010; Widmer et al. 2009; Rieseberg and Blackman 2010; Strasburg et al. 2012).

The genus *Epidendrum* L. is one of the most diverse genera in the Neotropical region, including a massive number of species (1500) distributed from northern Argentina to southern Florida (Hágsater and Soto-Arenas 2005). Many species have broad geographic distributions, occurring in different biomes and at different latitudes, making them interesting models for studying phylogeographic patterns across different plant communities (Hágsater and Soto-Arenas 2005; Pinheiro et al. 2011). Moreover, some species groups, such as those in *Epidendrum* subgenus *Amphiglottium*, are very easy to cultivate (Hágsater and Soto-Arenas 2005; Pinheiro et al. 2009aa) and produce a large number of flowers in each inflorescence, making them particularly useful for crossing experiments among and within species (Pansarin and Amaral 2008; Pinheiro et al. 2010). One such species is *Epidendrum denticulatum* Barb. Rodr., a perennial terrestrial orchid that occurs in southeastern and northeastern Brazil (Fig. 1a). This orchid grows in two main Neotropical biomes: the Brazilian Atlantic Forest (in sand dune and scrub vegetation along the coast) and the Cerrado domains in the inner part of the continent (in grassland surrounded by woody savanna vegetation; Pinheiro and Barros 2007). *E. denticulatum* follows a generalized pollination model by deceit, in which flowers are visited by several butterfly species, but no reward (nectar) is offered (Almeida and Figueiredo 2003). The species is self-compatible, but pollinators are necessary for pollen transfer (Almeida and Figueiredo 2003).

Here, we investigated the evolution of early stages of intrinsic postmating isolation among different populations and lineages of *E. denticulatum*, in a broad phylogeographic context. We analyzed highly informative nuclear and plastid markers to depict the demographic processes associated with lineage diversification across different vegetation domains. In addition, controlled

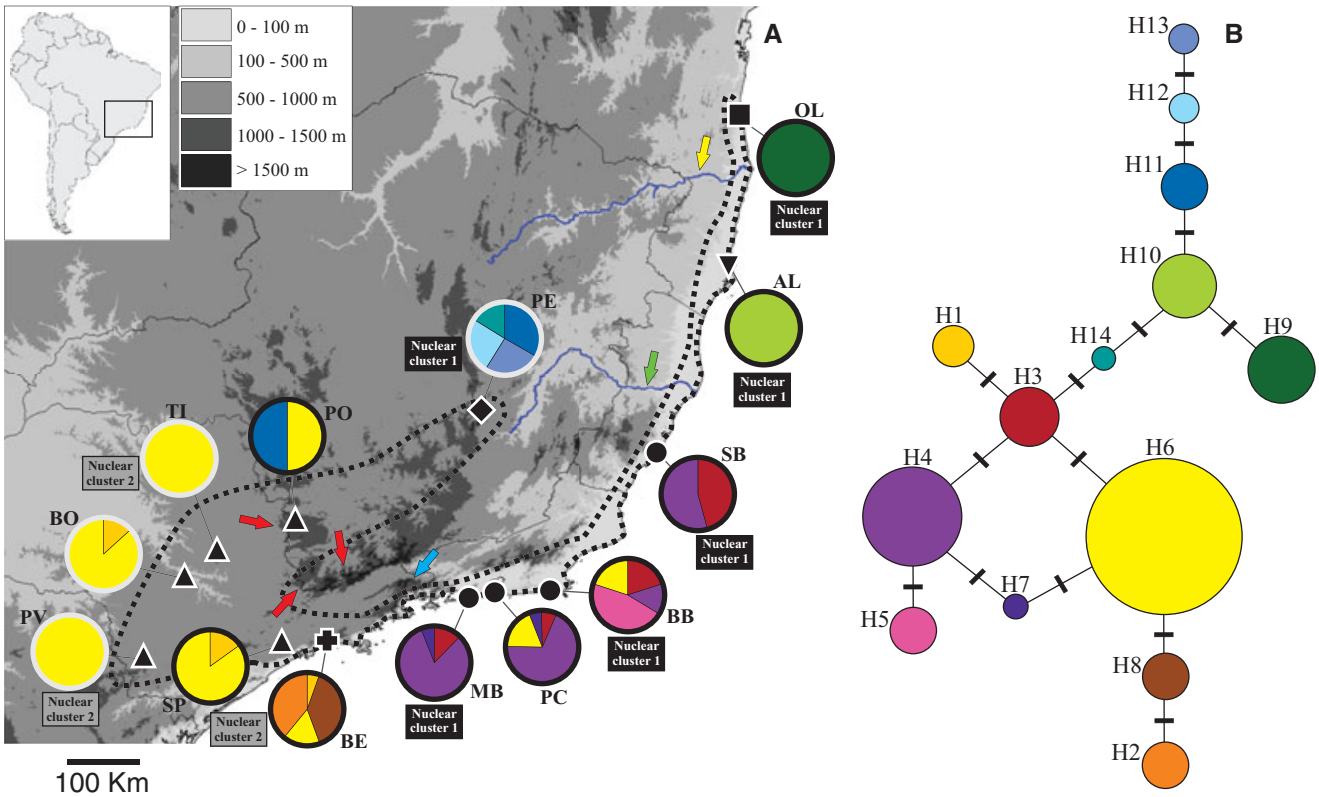

Figure 1. Map showing the current distribution of *Epidendrum denticulatum* in southeastern and northeastern Brazil, including sampled populations and genealogical relationships of the eight plastid DNA haplotypes recovered. (A) Pie charts reflect the frequency of occurrence of each haplotype in each population. Haplotype colors correspond to those shown in panel (B). Pie charts with gray and black outlines indicate the Cerrado and Brazilian Atlantic Forest populations, respectively. Nuclear genetic groups are indicated (nuclear cluster 1, black; nuclear cluster 2, gray). The dotted line delimits the geographical distribution of *Epidendrum denticulatum*. Different symbols (circles, triangles, squares, diamonds, and plus signs) indicate genetic groups identified by SAMOVA results. The Mantiqueira Range (red arrow), Bocaina Range (blue arrow), Doce River (green arrow), and Jequitinhonha River (yellow arrow) are indicated. (B) Statistical parsimony network linking the 14 haplotypes. Haplotypes are designated by numbers, and circle sizes are proportional to haplotype frequencies. The number of mutations required to explain transitions among haplotypes is indicated by cross hatches along the lines connecting the haplotypes.

crossing experiments were performed on individuals under cultivation (collected from different populations and regions) to understand the early stages of RI within this species. Specifically, we used this multidisciplinary approach to address the following questions: (1) Was the past distribution of *E. denticulatum* restricted to single versus multiple refugia during past climatic oscillations? (2) Do genetic diversity levels and demographic signatures differ among populations occurring in refuge and non-refuge regions, and are genetic disjunctions across the range of the species similar to those observed for other species in the same geographic range? (3) What is the current extent of genetic incompatibilities accumulated among divergent lineages, and are they related to the genetic structure observed? We discuss the phylogeography of *E. denticulatum* in light of fossil paleovegetation reconstruction, climate-based modeling, and other phylogeographic studies performed in the same geographic range, and examine the evolution of early stages of RI within this Neotropical plant species.

Materials and Methods

PLANT MATERIAL AND SAMPLING DESIGN

A total of 258 individuals across 13 populations (Fig. 1A; Table 1) spanning the entire range of *E. denticulatum* were collected, covering localities 50–1500 km apart and distributed in the Cerrado and Brazilian Atlantic Forest biomes (Fig. S1). For molecular analysis, leaf samples were cut into small pieces and transferred to silica gel for drying. Total genomic DNA was extracted as described by Pinheiro et al. (2008a).

MOLECULAR MARKERS AND GENOTYPING ASSAYS

Nine nuclear microsatellite markers were used. Five were isolated from *Epidendrum fulgens* (Eff26, Eff43, Eff45,

Table 1. Sampled populations of *Epidendrum denticulatum*, including the population name with their identification code, elevation above the sea level, habitat description, and vegetation domains (CER = Cerrado, BAF = Brazilian Atlantic Forest), and sample size analysed for nuclear and plastid markers.

				Sample size	
Population[1]	ID	Altitude (m)	Habitat/vegetation domain	Nuclear	Plastid
Olivença	OL	83	Shrubby Tabuleiro vegetation/BAF	21	16
Alcobaça	AL	4	Sand dune scrub vegetation/BAF	23	15
Setiba	SB	5	Sand dune scrub vegetation/BAF	20	13
Massambaba	BB	10	Sand dune scrub vegetation/BAF	22	15
Pão de Açúcar	PC	42	Coastal rock outcrop/BAF	–	16
Marambaia	MB	8	Sand dune scrub vegetation/BAF	18	16
Peti	PE	742	Open shrub vegetation/CER	23	12
Poços de Caldas	PO	1519	Forest on steep slopes/BAF	–	8
Itirapina	TI	687	Open shrub vegetation/CER	23	21
Botucatu	BO	469	Open shrub vegetation/CER	–	22
Bertioga	BE	2	Sand dune scrub vegetation/BAF	25	18
São Paulo	SP	815	Open shrub vegetation/BAF	–	13
Itapeva	PV	739	Open shrub vegetation/CER	24	16

[1]Populations occurring at historically stable areas (refuges) are in bold.

and Eff61; Pinheiro et al. 2008a Eff48, primer forward 5´-TGACCGTTTGAACCTTTTGGT-3´, reverse 5´-ATCCAGGCATGAGCAGCA-3´) and four were isolated from *Epidendrum puniceoluteum* Pinheiro and Barros (Epp18, Epp49, Epp86, and Epp96; Pinheiro et al. 2008bb). Six plastid loci were used: five plastid microsatellite loci (Epcp02, Epcp03, Epcp04, Epcp08, and Epcp09; Pinheiro et al. 2009b) and one single nucleotide polymorphism sequenced from the Epcp08 locus (Pinheiro et al. 2009b). All polymerase chain reaction amplifications were performed using an Applied Biosystems 2700 thermocycler (Applied Biosystems, Foster City, CA) following the protocol described by Pinheiro et al. (2008a). Microsatellite alleles were resolved on an ABI 3130 Genetic Analyzer automated sequencer and were sized with LIZ (500) standard using GENEMAPPER v. 3.7 software (Applied Biosystems).

GENETIC DIVERSITY OF THE SAMPLED POPULATIONS

The nuclear microsatellite diversity of nine populations was characterized using the number of alleles (*A*), number of private alleles (*PA*), allelic richness (*AR*), private allelic richness (*PAR*), expected (H_E) and observed (H_O) heterozygosity, and the inbreeding coefficient *f* (Weir and Cockerham 1984), calculated using the programs MSA v. 4.05 (Dieringer and Schloetterer 2003) and HP-RARE v. 1.0 (Kalinowski 2005). Departures of within-population inbreeding coefficients from Hardy–Weinberg equilibrium (HWE) were identified using exact tests in GENEPOP v. 4.0 (Raymond and Rousset 1995). The microsatellite dataset was tested for genotyping errors due to stuttering, short allele dominance, and null alleles using a Monte Carlo simulation of expected allele size differences using MICRO-CHECKER (Van Oosterhout et al. 2004).

All populations sampled were characterized for levels of diversity in plastid DNA markers. The number of haplotypes detected in each population, haplotype diversity, and haplotype richness were estimated with RAREFAC v. 3.5 software (Petit et al. 1998). Estimates of haplotype richness were corrected for differences in sample size using the rarefaction method.

NUCLEAR GENETIC STRUCTURING

Pairwise comparisons of F_{ST} between populations were estimated using the ARLEQUIN v. 3.5 program to estimate population genetic differentiation (Excoffier and Lischer 10). Genetic differentiation was also measured using the standardized genetic differentiation measure G'_{ST} (Hedrick 2005). Partitioning of genetic diversity was examined within populations, among populations, and between populations from the Brazilian Atlantic Forest (coastal) and Cerrado (inland), using analysis of molecular variance (AMOVA) implemented in the ARLEQUIN v. 3.5 software. The hypothesis that populations differentiated because of isolation-by-distance was tested by assessing the correlation between pairwise geographic distances with pairwise values of F_{ST} using a Mantel test in the GENEPOP v. 4.0 program. Correlation significance was estimated after performing 10,000 permutations between pairwise geographic distances and pairwise genetic differentiation matrices. To assess whether stepwise mutation had contributed to the population differentiation and phylogeographic structure ($R_{ST} > F_{ST}$), a test including 10,000 allele permutations was applied using the SPAGEDI v. 1.3d program (Hardy and Vekemans 2002).

Bayesian assignment analysis (in STRUCTURE v. 2.3.3; Hubisz et al. 2009) was used to assign individuals to genetic clusters (K) and estimate admixture proportions (Q) for each individual. A set of models was chosen in which individuals have admixed ancestries and correlated allele frequencies. The number of K was set from a minimum of one to a maximum of 10, and simulations were run for each K-value with a burn-in of 250,000 and 600,000 iterations each. The most probable number of genetic clusters (K) present in the data was defined following Evanno et al. (2005) using the Structure Harvester v. 6.0 program (Earl and von Holdt 2011).

PLASTID GENETIC STRUCTURING

The geographic structure of genetic variation in plastid DNA was investigated using three approaches. First, a median-joining network (Bandelt et al. 1999) was constructed based on plastid DNA to visualize the phylogenetic relationships among haplotypes using the NETWORK v. 4.5.1.0 program (www.fluxus-engineering.com). Second, a comparison of the distribution of diversity for ordered (R_{ST}) versus unordered (G_{ST}) haplotypes was carried out following Pons and Petit (1996) using the PERMUT / CpSSR program. To test for the presence of phylogeographic structure among populations (when R_{ST} is significantly larger than G_{ST}), 10,000 permutations of R_{ST} values were performed. Finally, a simulated annealing procedure was implemented using the SAMOVA 1.0 software (Dupanloup et al. 2002) to determine groups of populations that are geographically homogeneous and maximally differentiated from each other. The most likely number of groups (K) was determined by repeatedly running the software with 2–13 groups. The point at which the F_{CT} curve begins to plateau defines the optimal number of groups (Heuertz et al. 2004). The partitioning of genetic diversity within and among populations and between populations from the Brazilian Atlantic Forest (coastal) and Cerrado (inland) was assessed by AMOVA. In addition, pairwise comparisons of F_{ST} between populations were estimated using the ARLEQUIN v. 3.5 program.

DEMOGRAPHIC ANALYSES AND DIVERGENCE TIME ESTIMATES

Recent reductions in population size (i.e., genetic bottlenecks) were tested with nuclear markers based on M-ratios (Garza and Williamson 2001) using the ARLEQUIN v. 3.5 software. Significance for each population was assessed by comparing between M-ratios and critical values of M_C obtained by simulating the distribution of M-ratios under specific demographic and mutational conditions using the CRITICAL_M.EXE software (http://swfsc.noaa.gov/textblock.aspx?Division=FED&id=3298). The critical value M_C was set at the lower 5% tail of this distribution, and bottlenecks were detected when the M-ratio value was below the M_C threshold calculated. Different M_C values were simulated by modifying the parameter θ (0.5, 2.0, and 10.0) and the proportion of single-step mutations pg (0.1 and 0.3). The average size of non–one-step mutations (Δg) was kept constant at 3.5. Lower M_C values are more conservative because a bottleneck has to be of greater intensity to drop below this level (Garza and Williamson 2001), and, for this reason, the lower M_C value obtained was chosen to check bottleneck significance.

Plastid loci were used to calculate the average time of divergence of main network branches, using the time estimation routine implemented in the NETWORK program and the mutation rate for plastid microsatellites estimated by Cozzolino et al. (2003) for the orchid *Anacamptis palustris* (1.0×10^{-5}). Branch ages were measured based on haplotype H3, which was considered the main ancestral haplotype, and on descendent haplotypes H2, H5, H9, and H13, which are positioned at branch terminals (Fig. 1B), following the recommendations of Forster et al. (2004).

POPULATIONS IN STABLE VERSUS UNSTABLE CLIMATIC REGIONS

To test for the presence of higher genetic diversity at climate stable regions, the sampled populations were plotted on summary maps of historically stable areas published for the Cerrado (Werneck et al. 2012a) and Brazilian Atlantic Forest (Carnaval and Moritz 2008). The map published by Werneck et al. (2012a) represents the overlapped predicted outputs for four time projections: current, mid-Holocene (6 ka), Last Glacial Maximum (21 ka), and Last Interglacial (120 ka). The map published by Carnaval and Moritz (2008) represents the same time intervals, excluding the Last Interglacial. Only the Peti and Alcobaça populations were found to occur on refuge regions after examining the maps of historically stable areas (black areas of the map; Fig. 2). Thus, comparisons of genetic diversity levels were performed between refuge (Alcobaça and Peti) and nonrefuge (Olivença, Setiba, Massambaba, Pão de Açúcar, Marambaia, Poços de Caldas, Itirapina, Bertioga, São Paulo, Botucatu, and Itapeva) populations. The genetic parameters used for refuge and nonrefuge populations were private alleles, private allelic richness, allelic richness, expected heterozygozity, haplotype richness, and haplotype diversity. The same genetic parameters were also used for comparisons among populations from Brazilian Atlantic Forest (coastal) and Cerrado (inland). Statistical significance was assessed by the nonparametric Mann–Whitney test using SPSS 11.0 software (SPSS, Inc., Chicago, IL).

CROSSING EXPERIMENTS

F_1 seed viability was measured to evaluate the degree of postmating RI among nine populations of *E. denticulatum* (Olivença, Alcobaça, Peti, Massambaba, Pão de Açúcar, Marambaia, Bertioga, Itirapina, and Itapeva). Populations representing all plastid (six) and nuclear (two) genetic groups identified by

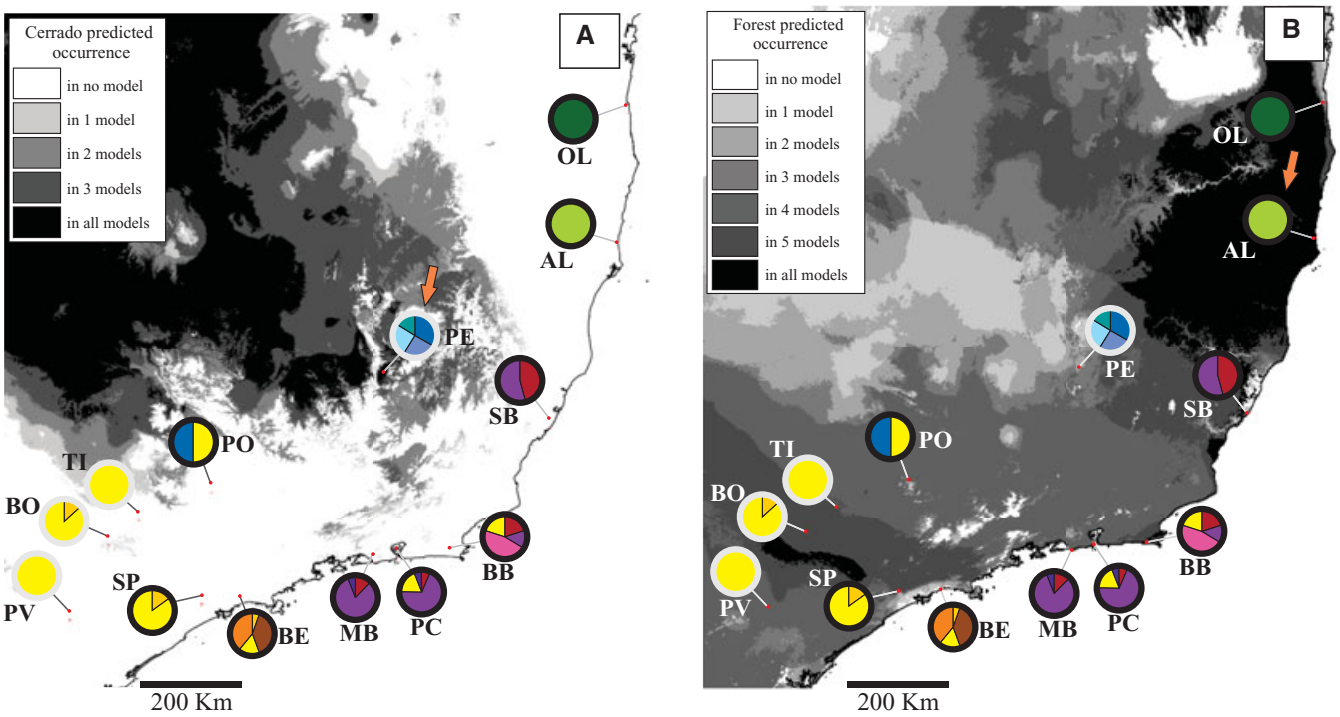

Figure 2. **Summary maps of historically stable areas for the Cerrado (A, modified from Werneck et al. 2012a) and Brazilian Atlantic Forest (B, modified from Carnaval and Moritz 2008) under broader definitions. The vegetation-predicted occurrence is indicated by gray tones, and stable areas (confirmed by all models) are in black. Sample sites are represented by small red dots. Pie charts reflect the frequency of occurrence of each haplotype in each population. Haplotype colors correspond to those shown in Figure 1B. Pie charts with gray and black outlines indicate the Cerrado and Brazilian Atlantic Forest populations, respectively. Orange arrows indicate populations occurring at historically stable areas in black (refuges).**

SAMOVA and STRUCTURE results, respectively, were chosen to explore the influence of genetic background on reproductive compatibility (Table S1). Controlled pollinations were performed on plants at the orchid collection of the Instituto de Botânica (São Paulo, Brazil) under similar conditions during 2010–2012. Two to eight plants were used in each intra- and interpopulation experiment (Table S1). In total, 154 flowers from 38 plants were used. All crosses were performed in both directions, each plant providing and receiving pollen. Intrapopulation pollinations were used as a control. Seeds were collected from mature capsules and stored at 4°C. Seed viability rates were evaluated using the tetrazolium test, following Pinheiro et al. (2010). The method was previously evaluated (Alvarez-Pardo et al. 2006; Suzuki et al. 2012) indicating that the percentage of seed viability, measured by tetrazolium test, was very similar to the respective percentage of seed germination for different orchid species. Samples of 200 seeds per fruit were analyzed with an optical microscope. The percentage of viable seeds was calculated by dividing the number of viable embryos by the total number of embryos scored. Seed viability data were obtained for each treatment and compared using the Mann–Whitney or Kruskal–Wallis tests, using the SPSS 11.0 software.

Postmating postzygotic isolation (P postzygotic) indices based on seed set results were calculated for interpopulation crosses following Scopece et al. (2007) and Jewell et al. (2012) using the formula: P postzygotic $= 1 -$ (% viable seeds in interpopulation crosses / % viable seeds in intrapopulation crosses). As all crosses were performed bidirectionally, reciprocal indices were averaged to provide a mean isolation index for each population pair. Genetic differentiation and geographic distance among populations were correlated with pairwise RI. For this, mean isolation indices were pooled to construct a matrix to be compared with matrices of nuclear pairwise F_{ST} and G'_{ST}, plastid F_{ST} and G_{ST}, and geographic distance, using the Mantel test in GENEPOP v. 4.0. To further assess the statistical significance of matrix comparisons, 10,000 permutations were performed.

Results

GENETIC DIVERSITY OF THE SAMPLED POPULATIONS

High levels of genetic diversity were observed for most populations at the nine sites genotyped with nuclear markers (Table 2). One to five private alleles were observed in almost all populations, and values of private allelic richness

Table 2. Genetic characterization of populations of *Epidendrum denticulatum*, including the populations sampled and biome of origin (CER = Cerrado; BAF = Brazilian Atlantic Forest), number of alleles (A), number of private alleles (PA), allelic richness (AR), private allelic richness (PAR), expected heterozygosity (H_E), observed heterozygosity (H_O), the within population inbreeding coefficient *f* and the *M*-ratio values for nine nuclear microsatellite loci, and the number of haplotypes (NH), haplotype richness (HR), and haplotype diversity (HD) for six plastid loci.

		Nuclear microsatellites loci								Plastid loci		
Populations/code[1]	Biome	A	PA	AR	PAR	H_E	H_O	f[2]	M-ratio[3]	NH	HR	HD
Olivença/OL	BAF	30	1	3.32	0.26	0.493	0.421	**0.149	0.620[4]	1	0.000	0.000
Alcobaça/AL	BAF	49	4	4.90	0.75	0.523	0.493	0.056	0.677	1	0.000	0.000
Setiba/SB	BAF	50	2	5.24	0.45	0.529	0.516	0.022	0.725	2	1.000	0.538
Massambaba/BB	BAF	40	0	4.20	0.30	0.501	0.451	0.101	0.667	4	2.646	0.733
Pão de Açúcar/PC	BAF	–	–	–	–	–	–	–		4	1.900	0.517
Marambaia/MB	BAF	40	2	4.37	0.37	0.513	0.460	0.103	0.685	3	1.267	0.342
Peti/PE	CER	41	1	4.19	0.49	0.429	0.423	0.021	0.654	4	2.871	0.803
Poços de Caldas/PO	BAF	–	–	–	–	–	–	–		2	1.000	0.571
Itirapina/TI	CER	38	1	3.85	0.23	0.476	0.331	***0.280	0.601[4]	1	0.000	0.000
Botucatu/BO	CER	–	–	–	–	–	–	–		2	0.764	0.247
Bertioga/BE	BAF	39	5	3.77	0.60	0.450	0.363	*0.197	0.610[4]	4	2.290	0.706
São Paulo/SP	BAF	–	–	–	–	–	–	–		2	0.872	0.282
Itapeva/PV	CER	27	0	2.85	0.15	0.395	0.374	0.039	0.562[4]	1	0.000	0.000

[1]Populations occurring at historically stable areas (refuges) are in bold;

[2]Departures of within-population inbreeding coefficients (*f*) from HWE are indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

[3]A population is considered to have undergone a bottleneck if its *M*-ratio value falls below the lower threshold of critical *M*-ratio (*M*c = 0.625) showed on Table 3.

[4]Populations where bottlenecks were detected.

were 0.15–0.75. The inbreeding coefficients were low, and only three populations(Olivença, Itirapina, and Bertioga) displayed significant departures from HWE due to heterozygote deficits. Genotyping errors and null alleles were ruled out by MICRO-CHECKER tests.

Fourteen haplotypes were detected for the six plastid regions analyzed (Fig. 1a; Table 2). A range of one to four haplotypes were found per population, which also showed extensive variation in both haplotype richness and diversity (Table 2).

GENETIC STRUCTURE REVEALED BY MOLECULAR MARKERS

Significant levels of nuclear genetic differentiation among populations ($P < 0.001$) were found for F_{ST} (0.168) and G'_{ST} (0.332). The F_{ST} values calculated for each pair of populations ranged from −0.057 to 0.309, and most values observed were significant ($P < 0.05$; Table S1). The lowest F_{ST} values were generally observed between adjacent populations (Table S1).

The Mantel test revealed that geographic distances were significantly correlated with nuclear genetic differentiation estimated by F_{ST} ($R^2 = 0.736$, $P < 0.0001$), indicating the presence of isolation by distance. The AMOVA results indicated that the main part of the genetic variation was partitioned within populations (87.45%, $P < 0.0001$), whereas only 12.55% of the nuclear diversity was explained among populations (Table 3). The differentiation between Brazilian Atlantic Forest and Cerrado populations was not significant ($P = 0.191$; Table 3). No sign of phylogeographic structure among populations was detected, as R_{ST} was not significantly larger than F_{ST} ($P > 0.05$).

Simulations performed in STRUCTURE consistently identified $K = 2$ clusters, as shown in Figure S2. The admixture proportion Q for each individual is shown in Figure 3. Almost all individuals from the coastal populations north of Bertioga and those of the inland Peti population showed strong assignment to cluster 1 ($Q < 0.95$), whereas specimens from the southernmost coastal population at Bertioga, and the inland populations Itirapina and Itapeva, showed admixture proportions strongly associated with cluster 2 ($Q > 0.95$).

The plastid marker analysis retrieved a well-resolved network (Fig. 1B), in which the most frequent haplotype (H6) was found in 39.8% of the individuals and in eight out of 13 sampled populations. Haplotypes H3, H4, H6, and H10 formed the core of the network topology and were distributed mainly along the coast. Haplotypes H12, H13, and H14 were restricted to the Peti population. Differentiation measures among all populations were

Table 3. Analysis of molecular variance (AMOVA) for nuclear and plastid microsatellite data using two different models, one with all populations pooled, and one with populations from different biomes (Cerrado and Brazilian Atlantic Forest) separated.

Source of variation	df	Variance components	Variation (%)	*P*-value
Nuclear microsatellite				
(1) Among populations	8	0.36023	16.68	$P < 0.001$
Within populations	389	1.79968	83.32	
(2) Between biomes (Cerrado and Brazilian Atlantic Forest)	1	2.80452	2.40	$P = 0.191$
Among populations within biomes	7	13.03463	11.17	$P < 0.001$
Within populations	389	100.82204	86.42	$P < 0.001$
Plastid microsatellite				
(1) Among populations	12	0.77260	75.14	$P < 0.001$
Within populations	188	0.25562	24.86	$P < 0.001$
(2) Between Biomes (Cerrado and Brazilian Atlantic Forest)	1	0.06601	6.22	$P = 0.203$
Among populations within biomes	11	0.73978	69.70	$P < 0.001$
Within populations	188	0.25562	24.08	$P < 0.001$

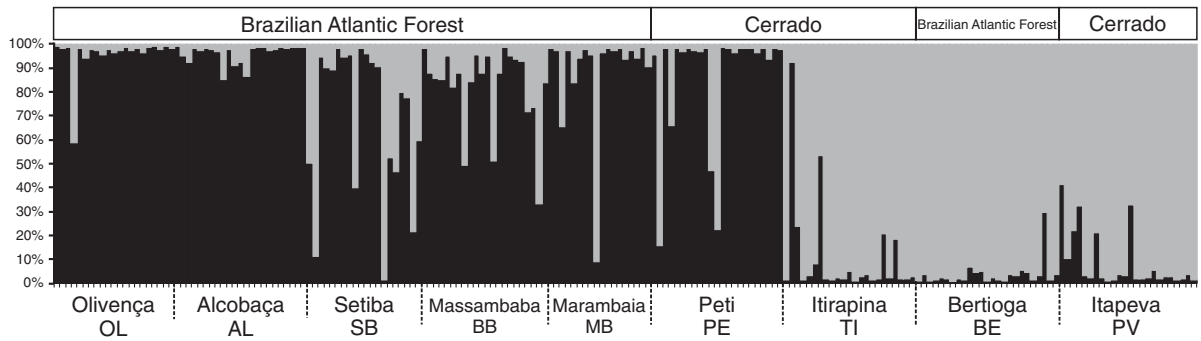

Figure 3. Summary of *Epidendrum denticulatum* population structure in southeastern and northeastern Brazil using Bayesian assignment analysis for a $K = 2$ population model. Cluster 1 (black) corresponds to coastal populations north of Bertioga, whereas specimens from the southernmost coastal population, Bertioga, and the inland populations Itirapina and Itapeva show admixture proportions strongly associated with cluster 2 (gray). Population codes and biome of origin (Brazilian Atlantic Forest and Cerrado) are indicated. See Table 1 for population details.

high, with $F_{ST} = 0.751$ and $G_{ST} = 0.572$. A clear sign of phylogeographic structure was detected among populations as genetic differentiation estimated with ordered haplotypes ($R_{ST} = 0.815$) was significantly higher ($P < 0.05$) than divergence for unordered haplotypes ($G_{ST} = 0.572$).

The SAMOVA results indicated six different genetic groups, as F_{CT} values increased progressively as K increased, reaching a plateau at $K = 6$. The composition of groups for $K = 6$ corresponded strongly to the geographic organization of haplotypes visually identified on the haplotype frequency map (Fig. 1A). According to the AMOVA results (Table 3), a high proportion of the genetic variability in the haplotype data resided among populations, and only 24.86% was attributed to within populations. The differentiation between the Brazilian Atlantic Forest and Cerrado populations was not significant ($P > 0.05$). Most pairwise F_{ST} values were significant ($P < 0.05$; Table S1), and low differentiation was generally observed between adjacent populations (Table S1).

POPULATION SIZE REDUCTION AND LINEAGE DIVERGENCE TIME

M-ratio values ranged from 0.562 to 0.725 across all sites and loci (Table 2). Considering the simulations performed with different values of θ and pg, the lowest critical M_C value observed was 0.625 (Table 4). At this *M*c threshold, the Olivença, Itirapina, Bertioga, and Itapeva populations showed signs of a past genetic bottleneck (Table 2). Furthermore, the *M*-ratios of these four populations were below the ad hoc bottleneck threshold value of $M_C = 0.680$ described by Garza and Williamson (2001).

The estimated divergence times indicated that all plastid lineages diversified during the Pleistocene, within the last million years (H3 and H2 = 552,631 ± 184,210; H3 and H5 = 451,923

Table 4. Parameters for the two-phased mutation model (TPM) used to generate critical values of *M*-ratio (*M*c). Theta (θ), proportion of single-step mutations (*pg*), and average size of non–one-step mutations (Δ*g*) were used to infer *M*c thresholds.

Parameters	θ	*pg*	Δ*g*	*M*c
1	0.5	0.1	3.5	0.797
2	0.5	0.3	3.5	0.646
3	2.0	0.1	3.5	0.748
4	2.0	0.3	3.5	0.625
5	10.0	0.1	3.5	0.737
6	10.0	0.3	3.5	0.664

± 390,460; H3 and H9 = 857,142 ± 285,714; H3 and H13 = 965,116 ± 525,576 years before present).

DIFFERENCES IN GENETIC DIVERSITY AMONG POPULATIONS IN REFUGE AND NONREFUGE REGIONS

Tests performed between refuge and nonrefuge populations did not support the hypothesis of higher genetic diversity in climate-stable regions. None of the genetic diversity parameters tested (private alleles, private allelic richness, allelic richness, H_E, haplotype diversity and haplotype richness) were significantly higher in refuge populations ($P > 0.05$). Similarly, significantly different genetic diversity levels were not found for most of the parameters tested ($P > 0.05$) between Brazilian Atlantic Forest (coastal) and Cerrado (inland) populations. Only H_E was significantly higher in the Brazilian Atlantic Forest populations ($P < 0.05$).

REPRODUCTIVE COMPATIBILITY

Reproductive isolating barriers were found among *E. denticulatum* populations, and significant seed viability asymmetry was detected, depending on which populations were crossed (Fig. 4; Table S1). Seed viability values for intrapopulational crosses, used as a control, were 77–94% and were similar across populations ($H = 9.874$, $P = 0.274$; Fig. 3). In contrast, interpopulational crosses were significantly different among populations ($H = 92.750$, $P < 0.001$), ranging from 0% to 96% seed viability. Seed viability among populations from different plastid genetic groups (TI × MB, TI × OL, TI × PE, MB × PE, MB × OL, PE × OL, TI × BE, TI × AL, MB × BE, MB × AL, OL × BE, OL × AL, PE × BE, PE × AL, BE × AL, TI × PC, see SAMOVA results in Fig. 1A) was significantly lower than among populations from the same plastid genetic group (PV × TI, MB × BB; $U = 1524.000$, $P < 0.005$). In contrast, no significant differences were observed between seed viability values from interpopulational crosses within (TI × PV, TI × BE, MB × BB, MB × PE, MB × OL, PE × OL, MB × AL, OL × AL, PE × AL) and among (TI × MB, TI × OL, TI × PE, TI × AL, MB × BE, OL × BE, PE × BE, BE × AL, TI × PC) nuclear genetic groups ($U = 3498.000$, $P = 0.623$). Significant asymmetric reproductive compatibility was found between populations MB × OL, OL × PE, OL × TI, OL × AL, OL × BE, TI × BE, and MB × BE ($P < 0.05$). Crosses between the TI and PC populations were significantly asymmetric only when individuals with different haplotypes (H6 and H7) were crossed (Fig. 4; Table S2). Low reproductive compatibility (0%) in both directions was only found between populations TI × PE and MB × AL (Fig. 4; Table S2).

Significant correlations were not observed when genetic differentiation (nuclear F_{ST} and G'_{ST}, plastid F_{ST} and G_{ST}) and geographic distance among populations were compared to mean RI indices from interpopulation crosses (Table S3). No significant correlations between mean RI indices and genetic distance or geographic distance were found using Mantel tests ($P > 0.05$).

Discussion

Recent glacial and interglacial periods have caused extinctions and repeated shifts in the ranges of taxa that survived (Behling 2002; Ledru et al. 2005; Bennett and Provan 2008), involving considerable demographic changes and providing opportunities for adaptation (Hewitt 2004; Skrede et al. 2008; Simon et al. 2009). A challenge for the study of speciation is to understand how climatically induced range changes have contributed to the evolution of reproductive barriers among formerly interbreeding populations (Hewitt 2001; Diniz-Filho et al. 2008; Avise 2009). Here, we report the combined use of phylogeographic tools and reproductive experiments to understand the evolution of early stages of lineage diversification and RI within a Neotropical orchid species. Most biogeographic inferences were based in the marked phylogeographic structure revealed by plastid markers. In contrast, nuclear markers showed low levels of genetic differentiation. Levels of genetic diversity were not associated with climate-stable regions, and the hypothesis of higher genetic diversity at refuges was not supported. Demographic oscillations (bottleneck signatures) were detected in populations at the range margins and in inland populations, indicating the influence of past climatic oscillations. Accordingly, divergence time estimates of the main plastid lineages fell within the Pleistocene, a period in which glacial / interglacial cycles caused great shifts in species distribution (Hewitt 2004). Seed viability was significantly lower in crosses among populations from different plastid genetic groups, which are geographically structured. Our results point to a combined action of forest and grassland expansion / fragmentation and ancient dispersal events that promoted differentiation among populations and shaped the early steps of RI in *E. denticulatum*.

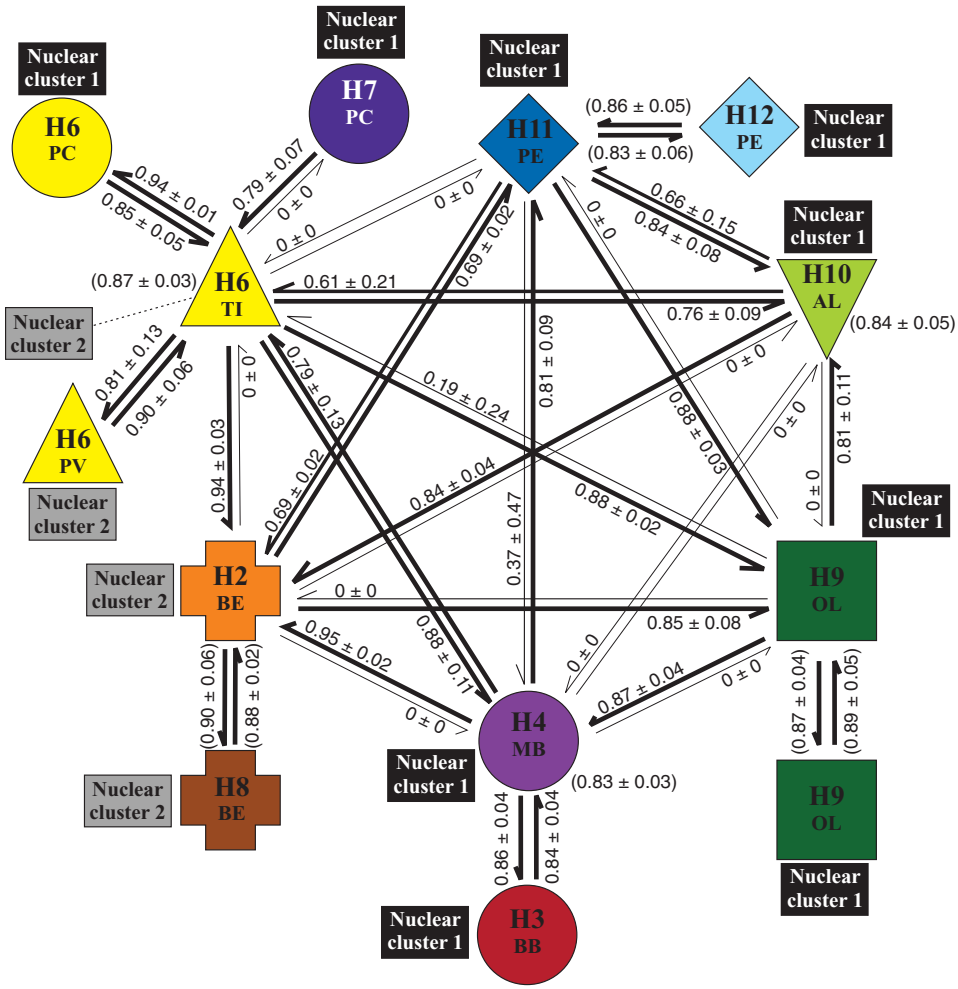

Figure 4. Reproductive compatibility among *Epidendrum denticulatum* individuals, based on seed viability following intra- and interpopulational experimental crosses. Letter-number codes indicate haplotypes, and symbols (triangles, circles, squares, and diamonds) correspond to the plastid genetic groups identified by SAMOVA (see Fig. 1A). Nuclear genetic groups are indicated (nuclear cluster 1, black; nuclear cluster 2, gray). Arrows point to the maternal parents in the crossing experiments. Thickness is proportional to cross-compatibility. Numbers between parentheses indicate seed set of intrapopulational crosses (mean ± standard deviation [SD]). Three specimens per population were used in the reciprocal crossing experiments; $H = 57.347$, df = 21, $P = 0.001$.

POPULATION STRUCTURE AND GENETIC DIVERSITY PATTERNS

Genetic structure was higher for plastid than for nuclear markers (Figs. 1a and 3), and this discrepancy in genetic structure among genomic compartments suggest that gene dispersal through pollen is higher than through seeds in this species. Although seeds of *Epidendrum* are wind dispersed, higher gene flow by pollen is normally observed in food-deceptive orchids (Cozzolino and Widmer 2005). This is because pollinators avoid plants in the same patch, which promotes pollen flow over long distances and reduces the chances of geitonogamous pollination (Chung et al. 254; Jacquemyn et al. 2006; Pinheiro et al. 2011).

E. denticulatum populations presented marked phylogeographic breaks, mainly in plastid markers and mostly associated with the Bocaina and Mantiqueira ranges, and the Doce and Jequitinhonha river basins (Fig. 1a). These geographic barriers have also been associated with current phylogeographic breaks for several animal (Costa 2003; Pellegrino et al. 2005; Cabanne et al. 2007; Tchaicka et al. 2007; Thomé et al. 2010) and plant species (Palma-Silva et al. 2009; Novaes et al. 2010; Turchetto-Zolet et al. 2012). The Mantiqueira and Bocaina ranges, and Doce and Jequitinhonha rivers are much older (65–23 Ma; Clapperton 1993; Lundberg et al. 1998; Ribeiro et al. 2011) than the divergence time estimated for organisms occurring in the same geographic region (Cabanne et al. 2007; Tchaicka et al. 2007; Novaes et al. 2010; Thomé et al. 2010). The divergence time calculated in such studies falls within the same time interval observed for divergence of the main *E. denticulatum* lineages, which took place in the last million years. For this reason, dispersal is a more plausible explanation than vicariance for the

origin of the plastid population structure. In fact, the hypothesis of a series of old dispersion events is supported by the sharing of the H6 ancestral haplotype between different genetic groups (Fig. 1A), the adjacent geographic distribution of closely related haplotypes (Fig. 1A,B), and the significant phylogeographic structure and isolation by distance detected.

Populations occurring in regions with stable climates mapped for the Brazilian Atlantic Forest (Carnaval and Moritz 2008) and Cerrado biomes (Werneck et al. 2012a) did not show significantly higher genetic diversity levels (populations AL and PE; Fig. 2), as expected for populations occurring in refuges (Bennett and Provan 2008; Carnaval and Moritz 2008; Carnaval et al. 2009). In fact, high levels of genetic diversity were observed for different and distant populations (Fig. 1a; Table 2), supporting the idea that *E. denticulatum* persisted in multiple refuges during interglacial periods (interglacial refugia; Bennett and Provan 2008). Phylogeographic studies have supported the existence of previously unknown and cryptic refugia for species associated with grassland and open vegetation communities (Jakob et al. 2009; Cosacov et al. 2010; Pinheiro et al. 2011). The Cerrado and Brazilian Atlantic Forest biomes are composed of a mosaic of different vegetation communities (Scarano 2002; Durigan 2006; Werneck 2011). Thus, current mapped refuges, resulting from climate modeling, may have limited power to identify refuges for species with different range characteristics (Porto et al. 2013). In fact, species distribution models have indicated different refuge regions for a wide array of taxa within the Brazilian Atlantic Forest (Thomé et al. 2010; Martins 2011; Porto et al. 2013). Moreover, recent studies have shown that the predictive power of climate modeling for refuge inference is weaker for species occurring in the southern portion of this biome (Thomé et al. 2010; Pinheiro et al. 2011; Porto et al. 2013). Thus, more empirical phylogeographic studies associated with species distribution models are needed for both the Cerrado and Brazilian Atlantic Forest biomes to explore the role of refuges in species diversification, mainly for species associated with grassland and open vegetation communities.

Haplotype sharing and nuclear genetic affinities were found between the southern Cerrado populations Itapeva, Botucatu, and Itirapina, and the Brazilian Atlantic Forest populations São Paulo and Bertioga (haplotypes H1 and H6; Figs. 1A and 3). Indeed, the AMOVA results did not indicate a significant divergence between Cerrado and Brazilian Atlantic Forest populations (Table 3). These results are in agreement with floristic data suggesting that the Cerrado and Brazilian Atlantic Forest biomes comprise a mixture of elements of various provenances and floristic affinities (Giulietti and Pirani 1988; Durigan 2006; Fiaschi and Pirani 2009). Extensive climatic oscillations occurred during the Middle and Late Pleistocene across both biomes (Behling and Lichte 1997; Behling 2002; Ledru et al. 2005). These changes also created vegetation mosaics with a mixed composition of forest and grassland communities (Behling and Negrelle 2001; Pessenda et al. 2009). Open scrub and grasslands expanded their geographical distributions with a decrease in temperature of 3–7°C during glacial cycles, displacing forest communities, both inland and lowlands close to the seashore, where tropical rain forest currently exists (Behling and Negrelle 2001; Behling 2002). This effect was particularly evident at range margins, where bottlenecks were detected (populations BE, PV, TI, and OL; Table 2), indicating recent population-reduction events (Table 2). Fragmentation increases population isolation and genetic drift becomes stronger mainly in peripheral populations, where the decrease in population size and genetic diversity is more pronounced (Eckert et al. 2008).

THE EVOLUTION OF REPRODUCTIVE ISOLATION AMONG LINEAGES

The significant phylogeographic structure found for plastid markers was congruent with the low levels of reproductive compatibility among populations and plastid genetic groups (Fig. 4; Tables S2 and S3). Outbreeding depression was found among different *E. denticulatum* populations and plastid genetic groups (Fig. 4), as significantly higher seed viability was observed for crosses performed among plants within populations and among individuals within the same plastid genetic groups (SAMOVA results; Figs. 3 and 4). In contrast, seed viability was significantly lower in crosses among individuals from different populations and from different plastid genetic groups (Fig. 4; Tables S2 and S3). The mean postzygotic isolation indices found between populations separated by the Doce River (AL × MB = 1.00), Jequitinhonha River (OL × AL = 0.52), Mantiqueira Range (PE × TI = 1.00), and Bocaina Range (MB × BE = 0.44) indicate the potential role of such phylogeographic barriers in the origin of early stages of RI. The accumulation of genetic incompatibilities by drift or adaptive divergence across multiple fragmented lineages may contribute substantially to the origin of reproductive barriers within species, as detected for other organisms (Lee 2000; Gómez et al. 2007; Skrede et al. 2008). Furthermore, these initial reproductive barriers were likely established recently, during the last million years, the period in which the main *E. denticulatum* lineages diverged.

Most interpopulation crosses returned asymmetric patterns of reproductive compatibility (Fig. 4; Table S2). Overall, seed viability was significantly lower in crosses among populations from different plastid genetic groups, but no significant difference was observed in seed viability between different nuclear genetic groups, suggesting the presence of negative cytonuclear interactions (Fig. 4; Table S2). For instance, cytonuclear conflict was particularly evident in crosses between the TI and PC populations (Fig. 4; Table S2). Crossing experiments performed between plants from the TI and PC populations with different nuclear assignments

but the same haplotypes resulted in higher seed set. In contrast, asymmetric compatibility was observed when individuals with different haplotypes were crossed (Fig. 4; Table S2). Asymmetries in both pre- and postzygotic RI stages can be associated with Bateson–Dobzhansky–Muller (BDM) genic incompatibilities involving uniparentally inherited genetic factors (Etterson et al. 2007; Turelli and Moyle 2007; Scopece et al. 2010; Greiner et al. 2011). Postmating–postzygotic negative interactions may evolve as a result of unidirectionally inherited genic changes, which generate cytonuclear genomic conflicts (Tiffin et al. 2001; Bomblies and Weigel 2007; Turelli and Moyle 2007; Greiner et al. 2011).

The polymorphic nature of RI barriers within *E. denticulatum* was confirmed by several crosses showing variable seed set results when the same population acted as pollen donor or pollen receptor (Fig. 4; Table S2). Furthermore, allele fixation was not supported as there was extensive variability in seed set results among individuals from the same population (Fig. 4; Table S2). The genetic polymorphism in RI loci among populations, or variable RI (VRI; reviewed by Cutter 2012) can be particularly evident at the intraspecific level during the earliest stages of speciation (Christie and Macnair 1987; Sweigart et al. 2006; Good et al. 2008). During early stages of divergence, incompatibility loci are expected to be polymorphic because this is the interval that corresponds to allele origin and fixation (Cutter 2012). Both local adaptation (Bomblies and Weigel 2007; Presgraves 2010) and genetic drift (Wade et al. 1999; Shuker et al. 2005) contribute to VRI, and the analysis of more nuclear loci coupled to reciprocal transplant experiments among populations from divergent habitats may clarify the origin of such incompatibilities.

Seed viability was significantly lower in crosses among different geographically structured plastid genetic groups. However, the intensity of RI was not proportional to the genetic differentiation between populations, as no significant correlations between mean RI indices and genetic or geographic distance were found. One possible explanation for lack of correlation could be the insufficient time to accumulate RI between lineages. Asymmetric RI was observed in most interpopulation crosses, suggesting incomplete barriers among most *E. denticulatum* populations (Fig. 4; Tables S2 and S3). The lack of correlation between genetic differentiation and RI may be a common pattern during early stages of RI and among recently derived taxa, as observed in other studies (Borba et al. 2001; Edmands 2002; Moyle et al. 2004; Scopece et al. 2007). In fact, Scopece et al. (2007, 2008) showed that postzygotic isolation in food-deceptive orchids increases according to the time elapsed because species divergence, suggesting the accumulation of incompatibility factors in many genes with small individual effects (Edmands 2002). Several different stages of RI can contribute to the overall RI observed, and extending experimental hybridization studies beyond the F_1 generation may reveal the specific isolating barrier associated with genetic differentiation (Ramsey et al. 2003, 2008; Lowry et al. 2008; Jewell et al. 2012).

Conclusions

The close relationship between outbreeding depression and the evolution of early stages of RI within species can help to disentangle the processes involved in the initial stages of speciation (Scopece et al. 2010). Phylogeographic surveys can offer a comprehensive genetic background for testing hypotheses associated with the evolution of RI among divergent lineages within species (Diniz-Filho et al. 2008; Scopece et al. 2010).

The phylogeographic survey performed with *E. denticulatum* allowed us to disentangle early stages of RI occurring at the population level. Multiple genetic diversity refuges were found, which were not associated with climate-stable regions modeled by recent studies. Bottlenecks occurred at range margins where successive cycles of forest expansion / contraction have been detected by other studies. Indeed, populations from the Brazilian Atlantic Forest and Cerrado biomes shared extensive genetic similarities, suggesting past floristic connections among these biomes. Reproductive experiments indicated high levels of outbreeding depression, probably achieved in a relatively short period of time, during the last million years. The diversity of seed viability results found for *E. denticulatum* clearly suggests polymorphic genetic mechanisms, possibly associated with many genes that have small individual effects. Crossing experiments with advanced hybrid generations (F_2 and backcrosses) are needed, as different barriers at different stages may contribute to the total RI within a species (reviewed by Scopece et al. 2010). Particularly important are reciprocal transplant experiments that can clarify the role of extrinsic forces driving early stages of RI, as selection for divergent habitats (Nosil et al. 2005; Sambatti et al. 2008). Although the connection between early stages of RI barriers and speciation events has rarely been shown in nonmodel plant species (Edmands 2002; Moyle et al. 2004; Lowry et al. 2008; Scopece et al. 2010), the permeability of these barriers and their variability within species in a phylogeographic context represents an interesting avenue for future research in the Neotropical region.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Pairwise comparisons of F_{ST} between populations of *Epidendrum denticulatum* in southeastern and northeastern Brazil based on nuclear (above diagonal) and plastid (below diagonal) microsatellites.

Table S2. Intrapopulation (7) and interpopulation (36) crossing treatments performed with 38 different *Epidendrum denticulatum* individuals (total of 154 crosses), and the proportion of viable seeds from each experiment.

Table S3. Mean postzygotic isolation indices based on seed set results from reciprocal crosses among different *Epidendrum denticulatum* populations.

Figure S1. Map showing the current distribution of *Epidendrum denticulatum* in southeastern and northeastern Brazil, including the main vegetation domains where populations were sampled.

Figure S2. Magnitude of ΔK from STRUCTURE analysis as a function of K, calculated according to the simulations described by Evanno et al. (2005).

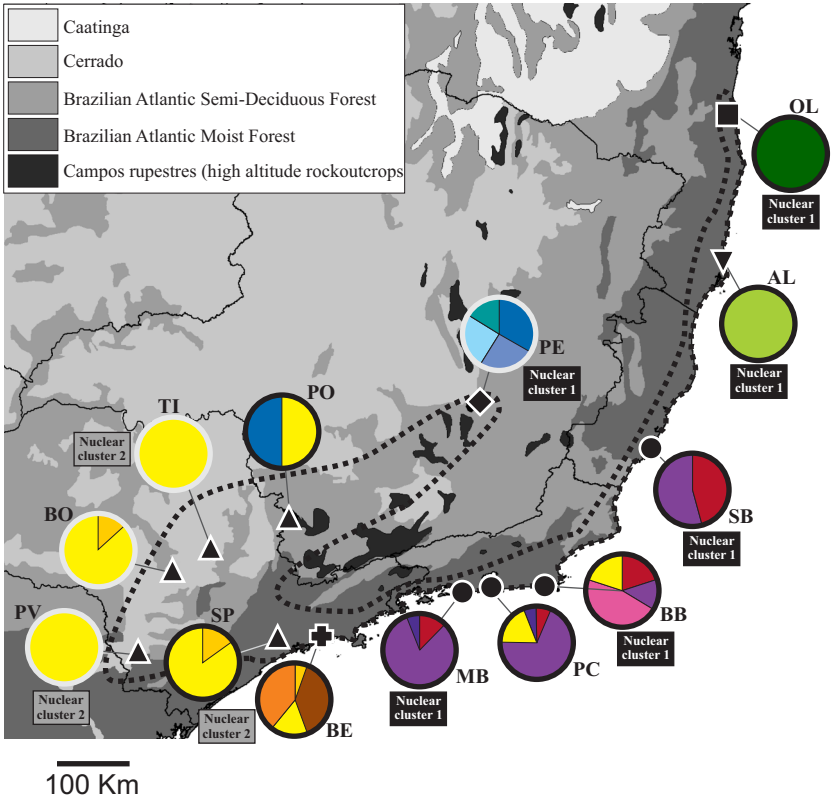

Figure S1. Map showing the current distribution of *Epidendrum denticulatum* in southeastern and northeastern Brazil, including the main vegetation domains where populations were sampled. The Brazilian Atlantic Semi-Deciduous Forest represent a transition between Brazilian Atlantic Forest and Cerrado biomes. Pie charts reflect the frequency of occurrence of each haplotype in each population. Pie charts with gray and black outlines indicate Cerrado and Brazilian Atlantic Forest populations, respectively. Nuclear genetic groups are indicated (Nuclear cluster 1 – black, Nuclear cluster 2 – gray). Different symbols (circle, triangle, square, diamond, plus) indicate genetic groups identified by SAMOVA results (see text).The dotted line delimits the geographical distribution of *E. denticulatum*.

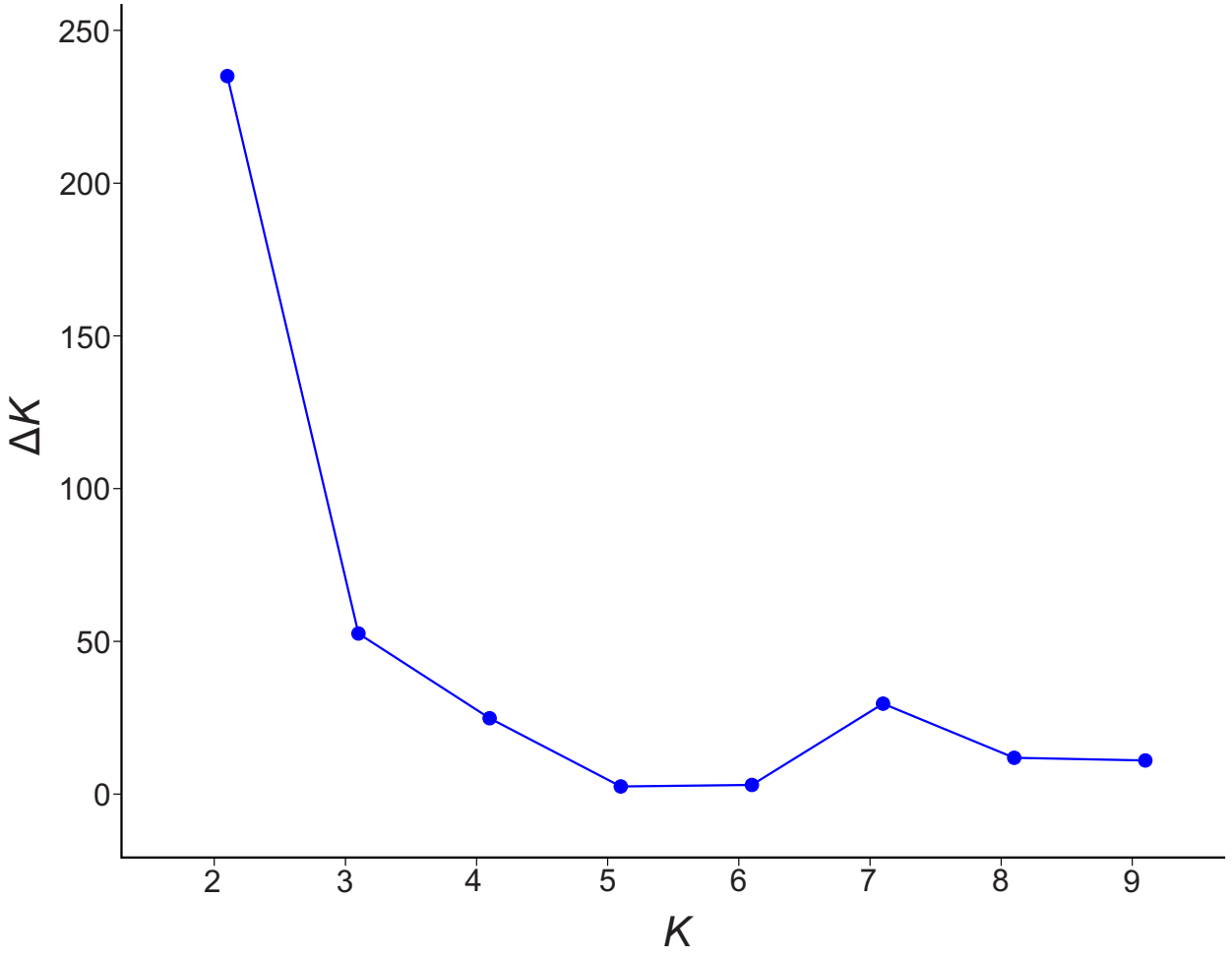

Figure S2. Magnitude of Δ*K* from STRUCTURE analysis as a function of *K* (details in Material and Methods), calculated according to the simulations described by Evanno *et al*. (2005). The modal value of these distributions indicates the true *K* or the uppermost level of structure, here two genetic clusters.

Table S1. Pairwise comparisons of F_{ST} between populations of *Epidendrum denticulatum* in southeastern and northeastern Brazil based on nuclear (above diagonal) and plastid (below diagonal) microsatellites. Dashes indicate populations that were not analysed with nuclear markers. See Table 1 for population identification.

	OL	AL	SB	BB	PC	MB	PE	PO	TI	BO	BE	SP	PV
OL	*	-0.001	0.001	**0.134**	-	**0.097**	**0.161**	-	0.021	-	**0.167**	-	**0.089**
AL	**1.000**	*	0.036	**0.157**	-	**0.115**	**0.143**	-	**0.072**	-	**0.236**	-	**0.056**
SB	**0.931**	**0.901**	*	-0.002	-	-0.033	**0.117**	-	-0.017	-	**0.061**	-	**0.153**
BB	**0.873**	**0.823**	**0.184**	*	-	-0.006	**0.246**	-	-0.020	-	**0.207**	-	**0.233**
PC	**0.900**	**0.862**	0.080	**0.222**	*	-	-	-	–	-	-	-	-
MB	**0.954**	**0.937**	0.164	**0.374**	0.024	*	**0.216**	-	-0.057	-	**0.178**	-	**0.160**
PE	**0.800**	**0.671**	**0.656**	**0.631**	**0.645**	**0.714**	*	-	**0.137**	-	**0.309**	-	**0.121**
PO	**0.801**	**0.676**	**0.492**	**0.384**	**0.483**	**0.636**	**0.350**	*	–	-	-	-	-
TI	**1.000**	**1.000**	**0.863**	**0.691**	**0.763**	**0.914**	**0.869**	**0.615**	*	-	**0.128**	-	**0.160**
BO	**0.926**	**0.898**	**0.667**	**0.509**	**0.598**	**0.762**	**0.775**	**0.391**	0.091	*	-	-	-
BE	**0.878**	**0.833**	**0.613**	**0.518**	**0.594**	**0.713**	**0.707**	**0.420**	**0.664**	**0.472**	*	-	**0.298**
SP	**0.934**	**0.907**	**0.641**	**0.462**	**0.562**	**0.753**	**0.739**	**0.320**	0.139	-0.063	**0.427**	*	-
PV	**1.000**	**1.000**	**0.841**	**0.654**	**0.733**	**0.901**	**0.847**	**0.561**	0.000	0.069	**0.629**	0.106	*

Values given in bold are significant at $P < 0.05$.

Table S2. Intra (7) and interpopulation (36) crossing treatments performed with 38 different *Epidendrum denticulatum* individuals (total of 154 crosses), and proportion of viable seeds from each experiment. Population codes followed by numbers indicate individual plants used for crosses. Nuclear genetic assignment (cluster 1, cluster 2) and plastid haplotype are indicated for each individual.

Crossing type	Origin of pollen donor	Nuclear cluster and haplotype	Origin of pollen recipient	Nuclear cluster and haplotype	Seed viability
Intrapopulation	Marambaia		Marambaia		
	MB-1	Cluster 1, H4	MB-3	Cluster 1, H4	0,79
	MB-1	Cluster 1, H4	MB-4	Cluster 1, H4	0,86
	MB-2	Cluster 1, H4	MB-4	Cluster 1, H4	0,89
	MB -2	Cluster 1, H4	MB-5	Cluster 1, H4	0,80
	MB-3	Cluster 1, H4	MB-1	Cluster 1, H4	0,80
	MB-3	Cluster 1, H4	MB-6	Cluster 1, H4	0,84
	MB-4	Cluster 1, H4	MB-1	Cluster 1, H4	0,86
	MB-4	Cluster 1, H4	MB-2	Cluster 1, H4	0,85
	MB-4	Cluster 1, H4	MB-6	Cluster 1, H4	0,78
	MB-5	Cluster 1, H4	MB-2	Cluster 1, H4	0,82
	MB-6	Cluster 1, H4	MB-3	Cluster 1, H4	0,82
	MB-6	Cluster 1, H4	MB-4	Cluster 1, H4	0,84
Intrapopulation	Itirapina		Itirapina		
	TI-1	Cluster 2, H6	TI-4	Cluster 2, H6	0,82
	TI-2	Cluster 2, H6	TI-3	Cluster 2, H6	0,89
	TI-3	Cluster 2, H6	TI-2	Cluster 2, H6	0,87
	TI-4	Cluster 2, H6	TI-1	Cluster 2, H6	0,91
Intrapopulation	Peti		Peti		
	PE-1	Cluster 1, H11	PE-6	Cluster 1, H12	0,77
	PE-2	Cluster 1, H11	PE-7	Cluster 1, H12	0,88
	PE-3	Cluster 1, H11	PE-5	Cluster 1, H12	0,78
	PE-4	Cluster 1, H11	PE-8	Cluster 1, H12	0,88
	PE-5	Cluster 1, H12	PE-3	Cluster 1, H11	0,90
	PE-6	Cluster 1, H12	PE-1	Cluster 1, H11	0,79
	PE-7	Cluster 1, H12	PE-2	Cluster 1, H11	0,90
	PE-8	Cluster 1, H12	PE-4	Cluster 1, H11	0,85
Intrapopulation	Olivença		Olivença		
	OL-1	Cluster 1, H9	OL-2	Cluster 1, H9	0,82
	OL-1	Cluster 1, H9	OL-3	Cluster 1, H9	0,86
	OL-2	Cluster 1, H9	OL-1	Cluster 1, H9	0,91
	OL-2	Cluster 1, H9	OL-3	Cluster 1, H9	0,84
	OL-3	Cluster 1, H9	OL-1	Cluster 1, H9	0,88
	OL-3	Cluster 1, H9	OL-2	Cluster 1, H9	0,94
Intrapopulation	Alcobaça		Alcobaça		
	AL-1	Cluster 1, H10	AL-2	Cluster 1, H10	0,78
	AL-2	Cluster 1, H10	AL-1	Cluster 1, H10	0,90
	AL-3	Cluster 1, H10	AL-4	Cluster 1, H10	0,80
	AL-4	Cluster 1, H10	AL-3	Cluster 1, H10	0,88

Table Sx: continued

Crossing type	Polen donor	Nuclear cluster and haplotype	Pollen recipient	Nuclear cluster and haplotype	Seed viability (%)
Intrapopulation	Bertioga		Bertioga		
	BE-1	Cluster 2, H2	BE-4	Cluster 2, H8	0.95
	BE-2	Cluster 2, H2	BE-5	Cluster 2, H8	0.94
	BE-3	Cluster 2, H2	BE-6	Cluster 2, H8	0.83
	BE-4	Cluster 2, H8	BE-1	Cluster 2, H2	0.91
	BE-5	Cluster 2, H8	BE-2	Cluster 2, H2	0.88
	BE-6	Cluster 2, H8	BE-3	Cluster 2, H2	0.87
Intrapopulation	Pão de Açúcar		Pão de Açúcar		
	PC-1	Cluster 1, H6	PC-2	Cluster 1, H6	0.88
	PC-2	Cluster 1, H6	PC-1	Cluster 1, H6	0.85
Interpopulation	Itirapina		Marambaia		
	TI-1	Cluster 2, H6	MB-1	Cluster 1, H4	0.67
	TI-2	Cluster 2, H6	MB-1	Cluster 1, H4	0.92
	TI-3	Cluster 2, H6	MB-2	Cluster 1, H4	0.95
	TI-3	Cluster 2, H6	MB -3	Cluster 1, H4	0.94
	TI-4	Cluster 2, H6	MB-3	Cluster 1, H4	0.93
Interpopulation	Marambaia		Itirapina		
	MB-1	Cluster 1, H4	TI-1	Cluster 2, H6	0.57
	MB-1	Cluster 1, H4	TI-2	Cluster 2, H6	0.90
	MB-2	Cluster 1, H4	TI-3	Cluster 2, H6	0.84
	MB -3	Cluster 1, H4	TI-3	Cluster 2, H6	0.79
	MB-3	Cluster 1, H4	TI-4	Cluster 2, H6	0.85
Interpopulation	Peti		Itirapina		
	PE-1	Cluster 1, H11	TI-1	Cluster 2, H6	0.00
	PE-2	Cluster 1, H11	TI-1	Cluster 2, H6	0.00
	PE-3	Cluster 1, H11	TI-2	Cluster 2, H6	0.00
	PE-4	Cluster 1, H11	TI-2	Cluster 2, H6	0.00
Interpopulation	Itirapina		Peti		
	TI-1	Cluster 2, H6	PE-1	Cluster 1, H11	0.00
	TI-1	Cluster 2, H6	PE-2	Cluster 1, H11	0.00
	TI-2	Cluster 2, H6	PE-3	Cluster 1, H11	0.00
	TI-2	Cluster 2, H6	PE-4	Cluster 1, H11	0.00
Interpopulation	Olivença		Itirapina		
	OL-1	Cluster 1, H9	TI-1	Cluster 2, H6	0.50
	OL-2	Cluster 1, H9	TI-2	Cluster 2, H6	0.29
	OL-2	Cluster 1, H9	TI-3	Cluster 2, H6	0.00
	OL-3	Cluster 1, H9	TI-4	Cluster 2, H6	0.00
Interpopulation	Itirapina		Olivença		
	TI-1	Cluster 2, H6	OL-1	Cluster 1, H9	0.89
	TI-2	Cluster 2, H6	OL-2	Cluster 1, H9	0.86
	TI-3	Cluster 2, H6	OL-2	Cluster 1, H9	0.85
	TI-4	Cluster 2, H6	OL-3	Cluster 1, H9	0.91

Table Sx: continued

Crossing type	Polen donor	Nuclear cluster and haplotype	Pollen recipient	Nuclear cluster and haplotype	Seed viability (%)
Interpopulation	Peti		Marambaia		
	PE-1	Cluster 1, H11	MB-3	Cluster 1, H4	0.00
	PE-2	Cluster 1, H11	MB-4	Cluster 1, H4	0.71
	PE-3	Cluster 1, H11	MB-4	Cluster 1, H4	0.79
	PE-4	Cluster 1, H11	MB-5	Cluster 1, H4	0.00
Interpopulation	Marambaia		Peti		
	MB-3	Cluster 1, H4	PE-1	Cluster 1, H11	0.66
	MB-4	Cluster 1, H4	PE-2	Cluster 1, H11	0.89
	MB-4	Cluster 1, H4	PE-3	Cluster 1, H11	0.83
	MB-5	Cluster 1, H4	PE-4	Cluster 1, H11	0.85
Interpopulation	Olivença		Marambaia		
	OL-1	Cluster 1, H9	MB-3	Cluster 1, H4	0.92
	OL-1	Cluster 1, H9	MB-4	Cluster 1, H4	0.83
	OL-1	Cluster 1, H9	MB-5	Cluster 1, H4	0.83
	OL-3	Cluster 1, H9	MB-6	Cluster 1, H4	0.88
Interpopulation	Marambaia		Olivença		
	MB-3	Cluster 1, H4	OL-1	Cluster 1, H9	0.00
	MB-4	Cluster 1, H4	OL-1	Cluster 1, H9	0.00
	MB-5	Cluster 1, H4	OL-1	Cluster 1, H9	0.00
	MB-6	Cluster 1, H4	OL-3	Cluster 1, H9	0.00
Interpopulation	Peti		Olivença		
	PE-3	Cluster 1, H11	OL-2	Cluster 1, H9	0.90
	PE-3	Cluster 1, H11	OL-3	Cluster 1, H9	0.84
	PE-4	Cluster 1, H11	OL-2	Cluster 1, H9	0.87
	PE-4	Cluster 1, H11	OL-3	Cluster 1, H9	0.92
Interpopulation	Olivença		Peti		
	OL-2	Cluster 1, H9	PE-3	Cluster 1, H11	0.00
	OL-3	Cluster 1, H9	PE-3	Cluster 1, H11	0.00
	OL-2	Cluster 1, H9	PE-4	Cluster 1, H11	0.00
	OL-3	Cluster 1, H9	PE-4	Cluster 1, H11	0.00
Interpopulation	Massambaba		Marambaia		
	BB-1	Cluster 1, H3	MB-1	Cluster 1, H4	0.90
	BB-2	Cluster 1, H3	MB-1	Cluster 1, H4	0.89
	BB-1	Cluster 1, H3	MB-2	Cluster 1, H4	0.89
	BB-2	Cluster 1, H3	MB -2	Cluster 1, H4	0.86
	BB-1	Cluster 1, H3	MB-3	Cluster 1, H4	0.77
	BB-2	Cluster 1, H3	MB-3	Cluster 1, H4	0.83
Interpopulation	Marambaia		Massambaba		
	MB-1	Cluster 1, H4	BB-1	Cluster 1, H3	0.88
	MB-1	Cluster 1, H4	BB-2	Cluster 1, H3	0.85
	MB-2	Cluster 1, H4	BB-1	Cluster 1, H3	0.84

Table Sx: continued

Crossing type	Polen donor	Nuclear cluster and haplotype	Pollen recipient	Nuclear cluster and haplotype	Seed viability (%)
Interpopulation	Marambaia		Massambaba		
	MB -2	Cluster 1, H4	BB-2	Cluster 1, H3	0.87
	MB-3	Cluster 1, H4	BB-1	Cluster 1, H3	0.77
	MB-3	Cluster 1, H4	BB-2	Cluster 1, H3	0.81
Interpopulation	Itapeva		Itirapina		
	PV-1	Cluster 2, H6	TI-2	Cluster 2, H6	0.92
	PV-2	Cluster 2, H6	TI-3	Cluster 2, H6	0.96
	PV-3	Cluster 2, H6	TI-4	Cluster 2, H6	0.84
Interpopulation	Itirapina		Itapeva		
	TI-2	Cluster 2, H6	PV-1	Cluster 2, H6	0.66
	TI-3	Cluster 2, H6	PV-2	Cluster 2, H6	0.86
	TI-4	Cluster 2, H6	PV-3	Cluster 2, H6	0.92
Interpopulation	Bertioga		Itirapina		
	BE-1	Cluster 2, H2	TI-2	Cluster 2, H6	0.00
	BE-2	Cluster 2, H2	TI-3	Cluster 2, H6	0.00
Interpopulation	Itirapina		Bertioga		
	TI-2	Cluster 2, H6	BE-1	Cluster 2, H2	0.92
	TI-3	Cluster 2, H6	BE-2	Cluster 2, H2	0.97
Interpopulation	Bertioga		Olivença		
	BE-1	Cluster 2, H2	OL-2	Cluster 1, H9	0.00
	BE-2	Cluster 2, H2	OL-3	Cluster 1, H9	0.00
Interpopulation	Olivença		Bertioga		
	OL-2	Cluster 1, H9	BE-1	Cluster 2, H2	0.91
	OL-3	Cluster 1, H9	BE-2	Cluster 2, H2	0.79
Interpopulation	Alcobaça		Bertioga		
	AL-2	Cluster 1, H10	BE-1	Cluster 2, H2	0.87
	AL-3	Cluster 1, H10	BE-3	Cluster 2, H2	0.81
Interpopulation	Bertioga		Alcobaça		
	BE-1	Cluster 2, H2	AL-2	Cluster 1, H10	0.00
	BE-3	Cluster 2, H2	AL-3	Cluster 1, H10	0.00
Interpopulation	Peti		Bertioga		
	PE-3	Cluster 1, H11	BE-1	Cluster 2, H2	0.68
	PE-4	Cluster 1, H11	BE-3	Cluster 2, H2	0.71
Interpopulation	Bertioga		Peti		
	BE-1	Cluster 2, H2	PE-3	Cluster 1, H11	0.78
	BE-3	Cluster 2, H2	PE-4	Cluster 1, H11	0.82
Interpopulation	Marambaia		Bertioga		
	MB-5	Cluster 1, H4	BE-2	Cluster 2, H2	0.93
	MB-6	Cluster 1, H4	BE-3	Cluster 2, H2	0.97
Interpopulation	Bertioga		Marambaia		
	BE-2	Cluster 2, H2	MB-5	Cluster 1, H4	0.00
	BE-3	Cluster 2, H2	MB-6	Cluster 1, H4	0.00

Table Sx: continued

Crossing type	Polen donor	Nuclear cluster and haplotype	Pollen recipient	Nuclear cluster and haplotype	Seed viability (%)
Interpopulation	Itirapina		Pão de Açúcar		
	TI-1	Cluster 2, H6	PC-1	Cluster 1, H6	0.93
	TI-2	Cluster 2, H6	PC-2	Cluster 1, H6	0.95
Interpopulation	Pão de Açúcar		Itirapina		
	PC-1	Cluster 1, H6	TI-1	Cluster 2, H6	0.89
	PC-2	Cluster 1, H6	TI-2	Cluster 2, H6	0.81
Interpopulation	Itirapina		Pão de Açúcar		
	TI-1	Cluster 2, H6	PC-3	Cluster 2, H7	0.00
	TI-2	Cluster 2, H6	PC-3	Cluster 2, H7	0.00
Interpopulation	Pão de Açúcar		Itirapina		
	PC-3	Cluster 2, H7	TI-1	Cluster 2, H6	0.74
	PC-3	Cluster 2, H7	TI-2	Cluster 2, H6	0.84
Interpopulation	Marambaia		Alcobaça		
	MB-1	Cluster 1, H4	AL-3	Cluster 1, H10	0.00
	MB-2	Cluster 1, H4	AL-4	Cluster 1, H10	0.00
Interpopulation	Alcobaça		Marambaia		
	AL-3	Cluster 1, H10	MB-1	Cluster 1, H4	0.00
	AL-4	Cluster 1, H10	MB-2	Cluster 1, H4	0.00
Interpopulation	Olivença		Alcobaça		
	OL-2	Cluster 1, H9	AL-3	Cluster 1, H10	0.89
	OL-3	Cluster 1, H9	AL-4	Cluster 1, H10	0.73
Interpopulation	Alcobaça		Olivença		
	AL-3	Cluster 1, H10	OL-2	Cluster 1, H9	0.00
	AL-4	Cluster 1, H10	OL-3	Cluster 1, H9	0.00
Interpopulation	Peti		Alcobaça		
	PE-1	Cluster 1, H11	AL-1	Cluster 1, H10	0.95
	PE-2	Cluster 1, H11	AL-2	Cluster 1, H10	0.85
	PE-3	Cluster 1, H11	AL-3	Cluster 1, H10	0.82
	PE-4	Cluster 1, H11	AL-3	Cluster 1, H10	0.74
Interpopulation	Alcobaça		Peti		
	AL-1	Cluster 1, H10	PE-1	Cluster 1, H11	0.55
	AL-2	Cluster 1, H10	PE-2	Cluster 1, H11	0.52
	AL-3	Cluster 1, H10	PE-3	Cluster 1, H11	0.79
	AL-3	Cluster 1, H10	PE-4	Cluster 1, H11	0.80
Interpopulation	Alcobaça		Itirapina		
	AL-1	Cluster 1, H10	TI-1	Cluster 2, H6	0,51
	AL-2	Cluster 1, H10	TI-2	Cluster 2, H6	0,46
	AL-3	Cluster 1, H10	TI-4	Cluster 2, H6	0,86
Interpopulation	Itirapina		Alcobaça		
	TI-1	Cluster 2, H6	AL-1	Cluster 1, H10	0,86
	TI-2	Cluster 2, H6	AL-2	Cluster 1, H10	0,76
	TI-4	Cluster 2, H6	AL-3	Cluster 1, H10	0,67

Table S3. Mean postzygotic isolation indices based on seed set results from reciprocal crosses among different *Epidendrum denticulatum* populations. See text for details regarding the postzygotic isolation index used.

	OL	AL	BB	PC[1]	PC[2]	MB	PE	TI	BE	PV
OL	*									
AL	0.52	*								
BB	-	-	*							
PC[1]	-	-	-	*						
PC[2]	-	-	-	-	*					
MB	0.49	1.00	0.01	-	-	*				
PE	0.48	0.10	-	-	-	0.29	*			
TI	0.38	0.19	-	0.00	0.53	0.01	1.00	*		
BE	0.51	0.51	-	-	-	0.44	0.13	0.46	*	
PV	-	-	-	-	-	-	-	0.00	-	*

[1]individuals from Pão de Açúcar population showing haplotype H6; [2]individuals from Pão de Açúcar population showing haplotype H7