

Phylogeography and genetic differentiation along the distributional range of the orchid *Epidendrum fulgens*: a Neotropical coastal species not restricted to glacial refugia

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ABSTRACT

Aim Phylogeographical studies in the Brazilian Atlantic Forest (BAF) have mostly included species associated with forest habitats, whereas taxa associated with grassland and sand-dune plant communities have so far been largely overlooked. This study examines the phylogeography of the orchid *Epidendrum fulgens*, which occurs on coastal sand dunes and granitic outcrops, in order to identify major genetic divergences or disjunctions across the range of the species and to investigate the genetic signatures of past range contractions and expansions.

Location Southern and south-eastern seashore vegetation along the BAF biome, and granitic and arenitic outcrops that occur in the subtropical grassland plant communities located south of the BAF.

Methods Nine nuclear and four plastid microsatellite loci were used to genotype 424 individuals from 16 populations across the distributional range of *E. fulgens*. For both sets of markers, we estimated genetic diversity and population differentiation, testing for a north–south gradient of genetic diversity. The plastid haplotype network and a Bayesian assignment analysis of nuclear markers were used to infer population structure. Past demographic changes were investigated using a coalescence approach.

Results A deep disjunction was found between northern populations within the BAF and southern populations outside the BAF that occur on granitic and arenitic outcrops. Recent demographic reductions were detected in northern populations on coastal sands. Such demographic changes were not expected for those populations, as previous studies with forest species had found evidence of population expansion in the same areas. Higher genetic diversity was found in southern populations on granite, in contrast to patterns observed in previous studies of forest species.

Main conclusions The results are consistent with the long-term persistence of *E. fulgens*. Bottlenecks were detected in populations from areas where population expansion events have been detected in other plant (and animal) species, suggesting that forest expansion after the Last Glacial Maximum played a role in the population fragmentation and decrease in genetic diversity in *E. fulgens*. A substantial genetic division in *E. fulgens* corresponds to the ‘Portal de Torres’, a region that demarcates the northern limits of subtropical grassland plant communities and the southern limits of the BAF.

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Keywords

Brazilian Atlantic Forest, *Epidendrum*, gene flow, genetic structure, microsatellites, Orchidaceae, phylogeography.

INTRODUCTION

The identification of historical demographic events and inference of population dynamics following the Last Glacial Maximum (LGM) are important for understanding the distribution of organismal diversity today (e.g. Hewitt, 1996). Because genetic signatures of population retraction and expansion depend on the geographical context, tests of demographic scenarios based on molecular markers have contributed greatly to current knowledge of biogeography (Diniz-Filho *et al.*, 2008). One of the main goals of phylogeographical studies is the identification of historically stable areas or refugia, where populations of many taxa survived during glacial periods (Carnaval *et al.*, 2009). Range contractions and fragmentation events have left genetic signatures in populations from refugial areas that are different from those in populations from re-colonized regions (Widmer & Lexer, 2001).

In South America, refugia have primarily been identified for species associated with forest environments, mainly because most phylogeographical studies have focused on organisms from core rain forest habitats, including both plants (Salgueiro *et al.*, 2004; Palma-Silva *et al.*, 2009; Ribeiro *et al.*, 2011) and animals (Costa, 2003; Cabanne *et al.*, 2007; Carnaval *et al.*, 2009; Thomé *et al.*, 2010). Using organisms not associated with forest habitats, a growing number of studies have shown that these species did not undergo dramatic range fragmentation in separate refugia during the LGM. Rather, species associated with grasslands (Jakob *et al.*, 2009; Cosacov *et al.*, 2010) and sand dunes (King *et al.*, 2009) are likely to have persisted in interconnected populations across most of their current distribution. Indeed, some species experienced population fragmentation after the LGM as a result of the expansion of forest elements (Ledru *et al.*, 2007; Antonelli *et al.*, 2010). This scenario suggests that a significant component of the evolutionary history of species not associated with forest habitats has been largely overlooked (Jakob *et al.*, 2009; King *et al.*, 2009; Cosacov *et al.*, 2010).

The Brazilian Atlantic Forest (BAF) is a biome of particular interest as it is a global biodiversity hotspot with approximately 20,000 plant species, corresponding to a considerable proportion of the South American biodiversity (Myers *et al.*, 2000). It is a mosaic of several plant communities, composed of the core BAF and communities at the periphery of the forests, such as swamp forests, inselberg vegetation and open scrub vegetation on the sandy coastal plains known as restinga (reviewed in Scarano, 2002). Pleistocene climatic oscillations had an impact on all plant communities within the BAF (Behling, 2002), and vegetation and climate reconstructions

based on pollen analysis provide strong evidence for forest contraction during the LGM (20–18 ka), with a concomitant expansion of grassland elements, mainly in southern and south-eastern Brazil (Behling & Negrelle, 2001; Behling, 2002). Recent phylogeographical studies of BAF taxa (Cabanne *et al.*, 2007; Ledru *et al.*, 2007; Carnaval *et al.*, 2009; Palma-Silva *et al.*, 2009; Ribeiro *et al.*, 2011) also confirm demographic changes consistent with responses to Pleistocene forest contractions and subsequent advances, after the LGM, into southern areas of the biome. In South America there have been few phylogeographical studies of taxa not associated with humid forest habitats, for example species from grasslands or sand dunes (but see Jakob *et al.*, 2009; Cosacov *et al.*, 2010), and the impact of the LGM on such Neotropical plant communities is yet to be fully explored.

In most angiosperms, plastid DNA is inherited maternally (Ennos, 1994; Petit *et al.*, 2005) and therefore provides a seed-specific marker, as used for analyses of orchids by Bateman *et al.* (2008), Fay *et al.* (2009) and Micheneau *et al.* (2010). In species in which seed flow is lower than pollen flow, it is predicted that the plastid genome will be highly structured when compared with nuclear genes (Petit *et al.*, 2005). Moreover, comparison with biparentally inherited nuclear markers has shed light on the relative importance of pollen- and seed-mediated gene flow in the structure of plant populations (Petit *et al.*, 2005; Duminil *et al.*, 2007). The distribution of genetic diversity in the nuclear and plastid genomes can be used to infer demographic processes in populations of plants. Natural history and empirical studies of many outcrossing plant species indicate that pollen movement is often the predominant form of gene flow (reviewed in Duminil *et al.*, 2007), but little is known about the dispersal abilities of species occupying unstable habitats, the demography of which resembles metapopulation models. When local demes undergo frequent extinction and recolonization events, as in the LGM, seed movement into empty patches of favourable habitat is the basic mechanism for the foundation of new subpopulations (Duminil *et al.*, 2007). The combined use of plastid and nuclear markers, in a phylogeographical context, can enhance the power to detect historical demographic fluctuations and help to disentangle the effects of pollen versus seed flow in shaping current population structure (King *et al.*, 2009; Palma-Silva *et al.*, 2009).

Epidendrum L. is the largest genus of Orchidaceae in the Neotropics, with about 1500 species distributed from the south-eastern United States to northern Argentina (Hágsater & Soto-Arenas, 2005). The genus contains many species with a wide distribution and high morphological diversity (Hágsater & Soto-Arenas, 2005). *Epidendrum fulgens* Brongn. is a

perennial terrestrial that occurs in the south-eastern region of the BAF and further south outside the BAF. This orchid grows in sand-dune fields and meadows (see Appendices S1 and S2 in Supporting Information), in the herbaceous and shrubby vegetation called restinga, on well-drained, post-beach Holocene sandy deposits (Scarano, 2002). In the southern part of its distribution, *E. fulgens* also occurs on granite-based bedrock and arenitic outcrops in the 'Depressão Central' physiographic region. *Epidendrum fulgens* is pollinated by butterflies, following a model of pollination by deceit, there being no reward (nectar) for the pollinators (Fuhro, 2006; Moreira *et al.*, 2008). The species is self-compatible, but pollinators are necessary for pollen transfer (Fuhro, 2006).

By using a species (*E. fulgens*) not associated with forest habitats as our model organism, we attempt to test for the existence of phylogeographical patterns different from those found in forest-dwelling species and to examine the population genetic dynamics in coastal regions, in the context of Late Quaternary climatic and vegetational changes. The distribution area of *E. fulgens* includes regions where the forest shows signs of both expansion and retraction, allowing us to assess whether genetic patterns correspond to *in situ* Pleistocene survival or to glacial retreat and post-glacial expansion. Furthermore, if *E. fulgens* has been submitted to a different demographic scenario, we expect that genetic analyses would be consistent with historical population stasis and long-term persistence in regions where forest species showed signs of fragmentation and isolation, as indicated by previous studies

in the same geographical range (Cabanne *et al.*, 2007; Palma-Silva *et al.*, 2009). Specifically, we use combined nuclear and plastid DNA data to address the following questions. (1) Was the current widespread distribution of *E. fulgens* stable during the Late Quaternary climatic oscillations? (2) Is there a north-south genetic divergence, or are there disjunctions across the range of the species similar to those observed for species associated with forest habitats? (3) What is the current extent of genetic structure, and which past demographic processes may have shaped any phylogeographical patterns? (4) What is the relative contribution of seed versus pollen flow to historical gene flow? We discuss the phylogeographical and genetic structure of *E. fulgens* in the light of palaeoclimate, vegetation reconstructions and the biogeographical history of the BAF.

MATERIALS AND METHODS

Plant material and sampling design

Sixteen populations (Fig. 1a and Table 1) spanning the entire range of *E. fulgens* were sampled, covering *c.* 1000 km. In total, 424 individuals across 16 populations were collected (Table 1). To avoid sampling the same individual twice, samples were collected at least 10 m apart. For molecular analysis, leaf samples were torn into small pieces and transferred into silica gel for drying. Total genomic DNA was extracted as described by Pinheiro *et al.* (2008a).

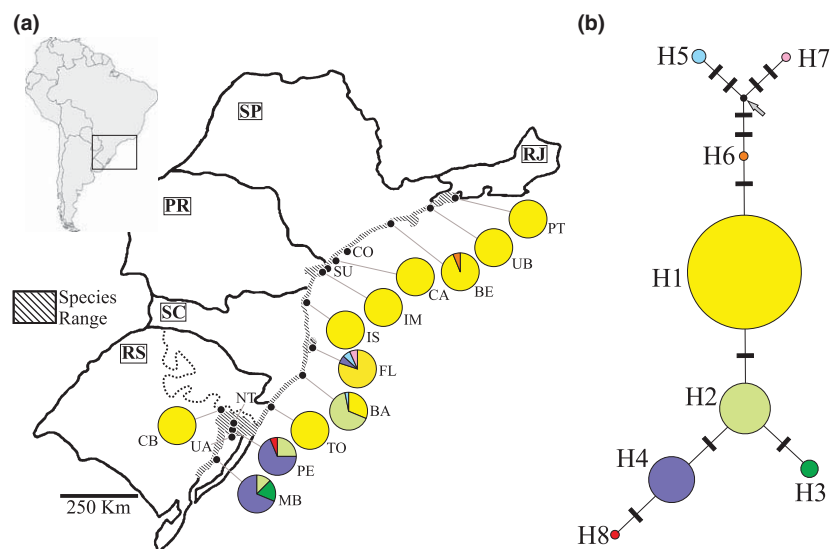


Figure 1 Map showing the current distribution of *Epidendrum fulgens* in southern and south-eastern Brazil, including populations sampled and genealogical relationships of the eight plastid DNA haplotypes recovered. (a) Pie charts reflect the frequency of occurrence of each haplotype in each population. Haplotype colours correspond to those shown in panel (b). The dotted line delimits the southern distribution of the Brazilian Atlantic Forest. (b) Statistical parsimony network linking the eight 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 along the lines connecting the haplotypes by the number of small bars. The arrow indicates a missing intermediate haplotype not found in the analysed individuals. Brazilian Federal States: RS, Rio Grande do Sul; SC, Santa Catarina; PR, Paraná; SP, São Paulo; RJ, Rio de Janeiro.

Table 1 Populations sampled with their identification code, geographical coordinates, elevation (m a.s.l.), habitat description and sample size analysed for nuclear and plastid markers of *Epidendrum fulgens* in southern and south-eastern Brazil. Populations are indicated as shown on the map in Fig. 1(a).

Population	ID	Latitude S	Longitude W	Elevation (m)	Habitat	Sample size	
						Nuclear	Plastid
Parati	PT	23°10′	44°40′	5	Sand-dune vegetation	34	16
Ubatuba	UB	23°22′	44°57′	16	Sand-dune vegetation	18	8
Bertioga	BE	23°46′	45°57′	11	Sand-dune vegetation	20	16
Ilha Comprida	CO	24°51′	47°42′	17	Sand-dune vegetation	20	–
Ilha do Cardoso	CA	25°04′	47°54′	8	Sand-dune vegetation	29	16
Ilha de Superagui	SU	25°27′	48°13′	9	Sand-dune vegetation	18	–
Ilha do Mel	IM	25°31′	48°17′	7	Sand-dune vegetation	24	16
Ilha de São Francisco	IS	26°16′	48°31′	16	Sand-dune vegetation	12	8
Florianópolis	FL	27°37′	48°27′	7	Sand-dune vegetation	52	15
Imbituba	BA	28°10′	48°41′	102	Sand-dune vegetation	50	32
Torres	TO	29°22′	49°45′	7	Sand-dune vegetation	25	16
Morro Santana	NT	30°03′	51°07′	293	Granitic rock outcrop	20	–
Morro São Pedro	PE	30°11′	51°06′	185	Granitic rock outcrop	21	16
Morro do Cabrito	CB	29°37′	51°39′	231	Arenitic rock outcrop	22	16
Itapuã	UA	30°21′	51°02′	89	Granitic rock outcrop	37	–
Arambaré	MB	30°54′	51°29′	8	Sand-dune vegetation	22	16
Overall						424	191

Molecular markers and genotyping assays

Nine nuclear microsatellite markers were used in this study, six isolated from *E. fulgens* (markers EFF26, EFF29, EFF43, EFF45, EFF61, EFF70; Pinheiro *et al.*, 2008a) and three isolated from *E. puniceoluteum* Pinheiro & Barros (markers Epp10, Epp18, Epp86; Pinheiro *et al.*, 2008b). Four plastid microsatellite loci (Epcp02, Epcp04, Epcp08, Epcp09; Pinheiro *et al.*, 2009) were used for identifying and characterizing plastid DNA haplotypes. All polymerase chain reaction (PCR) amplifications were performed in an Applied Biosystems 2700 thermocycler (Applied Biosystems, Foster City, CA, USA) following the protocol described by Pinheiro *et al.* (2008a). The same conditions were used for all loci to maximize standardization. Microsatellite alleles were resolved on an ABI 3130 Genetic Analyzer automated sequencer and were sized with LIZ (500) standard using GENEMAPPER 3.7 software (Applied Biosystems).

Genetic diversity of sampled populations

The nuclear microsatellite diversity of each population was characterized using the number of alleles (A), number of private alleles (PA), allelic richness (AR), expected (H_E) and observed (H_O) heterozygosity, and the inbreeding coefficient f (Weir & Cockerham, 1984), calculated using the programs FSTAT 2.9.3.2 (Goudet, 1995) and MSA 4.05 (Dieringer & Schlötterer, 2003). Departures from the Hardy–Weinberg equilibrium (HWE) for each population were identified using exact tests in GENEPOP 4.0 (Raymond & Rousset, 1995). The microsatellite data were tested for genotyping errors resulting from stuttering, short allele dominance and null alleles using a

Monte Carlo simulation of expected allele-size differences implemented in MICRO-CHECKER 2.2.3 (van Oosterhout *et al.*, 2004).

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

Biparental (nuclear) genetic structuring

Genetic differentiation was measured across all populations using the fixation index θ (Weir & Cockerham, 1984), the unbiased estimator of relative differentiation G_{ST} (Nei & Chesser, 1983) and the standardized genetic differentiation measure G'_{ST} (Hedrick, 2005). Pairwise comparisons of F_{ST} between populations were estimated using the program ARLEQUIN 3.5 (Excoffier & Lischer, 2010). Partitioning of genetic diversity within and among populations was examined by analysis of molecular variance (AMOVA) implemented in the software ARLEQUIN 3.5.

The hypothesis that populations are differentiated because of isolation-by-distance was tested by assessing the correlation between pairwise geographical distances with pairwise values of θ , G_{ST} and G'_{ST} using a Mantel test in the program FSTAT. A total of 10,000 random permutations were performed. In addition, allelic richness and gene diversity were tested against latitude using Spearman's rank correlation coefficient to determine if there is a north–south pattern of genetic diversity. The relationships between latitude and genetic parameters

were tested by linear, quadratic and cubic regression models, and the most likely model was selected based on the highest percentage variance explained ($PVE = R^2$).

Bayesian assignment analysis (in *STRUCTURE* 2.3.3; Hubisz *et al.*, 2009) was used to assign individuals to genetic clusters (K) and estimate admixture proportions (Q) for each individual. Following the new model developed for *STRUCTURE*, information of sampling localities was considered in the assignment tests. A set of models was chosen in which individuals had admixed ancestries and correlated allele frequencies. The number of K was set from a minimum of one to a maximum of ten, and two simulations were run for each K -value with a burn-in of 100,000 and 300,000 iterations, respectively. To define the most probable number of genetic clusters (K) present in the data, we used the method proposed by Evanno *et al.* (2005), which is based on an *ad hoc* measure ΔK that evaluates the second-order rate of change of the likelihood function with respect to K . Replicated runs were performed for each of the 10 models ($K = 1-10$) a minimum of three times to ensure consistent natural log probabilities.

Maternal (plastid) genetic structuring

A median-joining (MJ) network (Bandelt *et al.*, 1999) was constructed based on plastid DNA to visualize the relationships among haplotypes, using the program *NETWORK* 4.5.1.0 (<http://www.fluxus-engineering.com>). It uses maximum parsimony to reconstruct all possible shortest, least complex phylogenetic trees. Pairwise comparisons of F_{ST} between populations were calculated, and AMOVA was conducted using the software *ARLEQUIN*. Genetic differentiation was also measured by F_{ST} and G_{ST} , using the program *PERMUT/CpSSR* 2.0 (Pons & Petit, 1996). To search for a north-south gradient of genetic diversity, haplotype richness and haplotype diversity were tested against latitude, using the same procedure as for the nuclear markers.

Bottleneck tests

Recent population size reductions (i.e. genetic bottlenecks) were tested based on heterozygosity excess using the coalescent approach implemented in the program *BOTTLENECK* 1.2.02 (Cornuet & Luikart, 1996). The analysis was carried out under the two-phase model (TPM), which is known to fit microsatellite evolution better than other methods (Dirienzo *et al.*, 1994), with 95% contribution from the stepwise mutation model (or strict single-step mutations) and 5% contribution from the multiple-step mutations, for which the variance for mutation size was set to 12 as suggested in the user's manual. Statistical significance was assessed by 10,000 replications using a one-tailed Wilcoxon signed-rank test of mutation (M). The M -statistic values were calculated for each population according to Garza & Williamson (2001) to detect reductions in effective population size, using the software *ARLEQUIN*. Significance was assessed by comparison between the mean value M across all loci and the value $M = 0.680$, the threshold value

below which a population can reasonably be assumed to have undergone a reduction in population size (Garza & Williamson, 2001).

Gene flow by pollen and seed dispersal

The combined analysis of nuclear and plastid genomes allowed inferences to be made regarding the relative levels of gene flow by pollen and by seed, by comparing G_{ST} values from biparentally inherited nuclear markers (G_{STb}) with those from uniparentally inherited plastid markers (G_{STm}) (Ennos, 1994; Petit *et al.*, 2005). The relative contribution of pollen versus seed flow to total gene flow was estimated following Ennos (1994) and Petit *et al.* (2005), using equation 1 presented by Petit *et al.* (2005):

Pollen flow/seed flow

$$= [(1/G_{STb} - 1)(1 + F_{IS}) - 2(1/G_{STm} - 1)] / (1/G_{STm} - 1).$$

RESULTS

Genetic variation at sampled populations

For the 16 sites genotyped with nuclear markers, high levels of genetic diversity were observed for all genetic parameters (Table 2). The number of alleles ranged from 49 to 117, and the allelic richness ranged from 4.49 to 6.94. The expected and observed heterozygosity per population ranged from 0.597 to 0.761 and from 0.454 to 0.720, respectively. One to four private alleles were observed in almost all populations. The inbreeding coefficients were low in most populations, ranging from -0.004 to 0.245. Only five populations (Bertioga, Ilha Comprida, Imbituba, Morro São Pedro and Itapuã) displayed significant departures from HWE owing to heterozygote deficits. Genotyping errors, such as null alleles, were ruled out by *MICRO-CHECKER* tests.

For the four plastid microsatellite loci, we detected 8 haplotypes from 12 sampling locations (Fig. 1a; Table 2). Haplotype richness ranged from 0.000 to 1.667, and haplotype diversity ranged from 0.000 to 0.508. Seven out of 12 populations sampled for plastid markers had a fixed haplotype (H1).

Genetic structure and population differentiation revealed by nuclear markers

Low levels of differentiation across populations were found for θ (0.067), G_{ST} (0.097) and G'_{ST} (0.297). The F_{ST} values calculated for each pair of populations ranged from -0.033 to 0.377, and most values observed were significant ($P < 0.05$; Table 3). Generally, the lowest F_{ST} values were observed between adjacent populations (Table 3).

Geographical distances were significantly correlated with nuclear genetic differentiation as estimated by θ ($R^2 = 0.359$, $P < 0.0001$), G_{ST} ($R^2 = 0.496$, $P < 0.0001$) and G'_{ST} ($R^2 = 0.533$, $P < 0.0001$), suggesting the presence of isolation-by-

Table 2 Characterization of genetic variability in *Epidendrum fulgens* populations in southern and south-eastern Brazil. The number of alleles (*A*), number of private alleles (*PA*), allelic richness (*AR*), expected (H_E) and observed (H_O) heterozygosity and the within-population inbreeding coefficient *f* were estimated from nine nuclear microsatellite loci for 424 individuals. The number of haplotypes (*NH*), haplotype richness (*HR*) and haplotype diversity (*HD*) were estimated from four plastid microsatellite loci for 191 specimens. See Table 1 for sample size details for each population.

Population/Code	Nuclear microsatellites						Plastid microsatellites		
	<i>A</i>	<i>PA</i>	<i>AR</i>	H_E	H_O	<i>f</i>	<i>NH</i>	<i>HR</i>	<i>HD</i>
Parati/PT	64	4	4.63	0.622	0.573	0.080	1	0.000	0.000
Ubatuba/UB	49	0	4.49	0.599	0.574	0.042	1	0.000	0.000
Bertioga/BE	63	2	5.17	0.597	0.454	0.245***	2	0.500	0.125
Ilha Comprida/CO	77	0	5.26	0.686	0.616	0.105*	–	–	–
Ilha do Cardoso/CA	76	1	5.48	0.601	0.580	0.036	1	0.000	0.000
Ilha de Superagui/SU	77	1	6.36	0.652	0.593	0.094	–	–	–
Ilha do Mel/IM	74	0	5.78	0.644	0.573	0.113	1	0.000	0.000
Ilha de São Francisco/IS	69	2	6.39	0.688	0.650	0.057	1	0.000	0.000
Florianópolis/FL	117	2	6.94	0.722	0.686	0.050	4	1.600	0.371
Imbituba/BA	106	3	6.89	0.761	0.689	0.096***	3	1.220	0.486
Torres/TO	98	3	6.81	0.723	0.702	0.029	1	0.000	0.000
Morro Santana/NT	62	1	5.42	0.694	0.630	0.095	–	–	–
Morro São Pedro/PE	76	2	6.10	0.736	0.632	0.144**	3	1.462	0.492
Morro do Cabrito/CB	69	2	5.49	0.697	0.638	0.087	1	0.000	0.000
Itapuã/UA	88	2	6.09	0.702	0.627	0.109***	–	–	–
Arambaré/MB	66	1	5.51	0.717	0.720	–0.004	3	1.667	0.508

Departures from Hardy–Weinberg equilibrium, with Bonferroni correction, are indicated by asterisks (* $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$).

Table 3 F_{ST} values for pairwise comparison between populations of *Epidendrum fulgens* in southern and south-eastern Brazil based on nuclear (below diagonal) and plastid (above diagonal) microsatellites. Dashes indicate populations that were not analysed with plastid markers. See Table 1 for population identification.

	PT	UB	BE	CO	CA	SU	IM	IS	FL	BA	TO	NT	PE	CB	UA	MB
PT	*	0.000	0.000	–	0.000	–	0.000	0.000	0.069	0.484	0.000	–	0.859	0.000	–	0.791
UB	0.135	*	–0.050	–	0.000	–	0.000	0.000	0.004	0.423	0.000	–	0.816	0.000	–	0.731
BE	0.036	0.029	*	–	0.000	–	0.000	–0.050	0.037	0.461	0.000	–	0.825	0.000	–	0.760
CO	0.041	0.034	0.016	*	–	–	–	–	–	–	–	–	–	–	–	–
CA	–0.002	0.084	0.046	0.015	*	–	0.000	0.000	0.069	0.484	0.000	–	0.859	0.000	–	0.791
SU	0.056	0.032	0.025	0.002	0.012	*	–	–	–	–	–	–	–	–	–	–
IM	0.005	0.062	0.118	0.031	0.008	–0.009	*	0.000	0.069	0.484	0.000	–	0.859	0.000	–	0.791
IS	0.035	0.121	0.149	0.115	0.038	0.032	0.032	*	0.004	0.423	0.000	–	0.816	0.000	–	0.731
FL	0.060	0.106	0.106	0.071	0.029	0.033	0.035	0.010	*	0.281	0.069	–	0.580	0.069	–	0.529
BA	0.047	0.134	0.111	0.087	0.023	0.057	0.057	0.020	–0.004	*	0.484	–	0.485	0.484	–	0.440
TO	0.123	0.115	0.167	0.135	0.093	0.058	0.076	0.011	0.021	0.044	*	–	0.859	0.000	–	0.791
NT	0.169	0.170	0.228	0.175	0.147	0.071	0.133	0.071	0.055	0.099	0.039	*	–	–	–	–
PE	0.128	0.126	0.157	0.111	0.112	0.033	0.092	0.057	0.062	0.099	0.052	–0.004	*	0.859	–	–0.001
CB	0.140	0.128	0.189	0.132	0.114	0.030	0.091	0.054	0.037	0.076	0.038	–0.012	0.012	*	–	0.791
UA	0.175	0.157	0.216	0.181	0.139	0.099	0.143	0.027	0.001	0.045	–0.033	–0.002	0.024	–0.021	*	–
MB	0.282	0.311	0.377	0.331	0.273	0.223	0.257	0.140	0.118	0.171	0.069	0.053	0.105	0.062	0.040	*

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

distance. The distribution of genetic diversity across the range of the sampled populations was negatively correlated with latitude (allelic richness, $P < 0.05$; H_E , $P < 0.0001$). The relationship between genetic diversity and latitude was best described by the quadratic model (allelic richness, $R^2 = 0.755$; H_E , $R^2 = 0.738$). The model indicated higher values for the

mid-southern populations Florianópolis, Imbituba and Torres, with a tendency to decrease in both directions towards the range margins (Fig. 2a,b).

Simulations performed in STRUCTURE consistently identified $K = 2$ clusters, as show in Appendix S3. The admixture proportions Q for each individual are shown in Fig. 3.

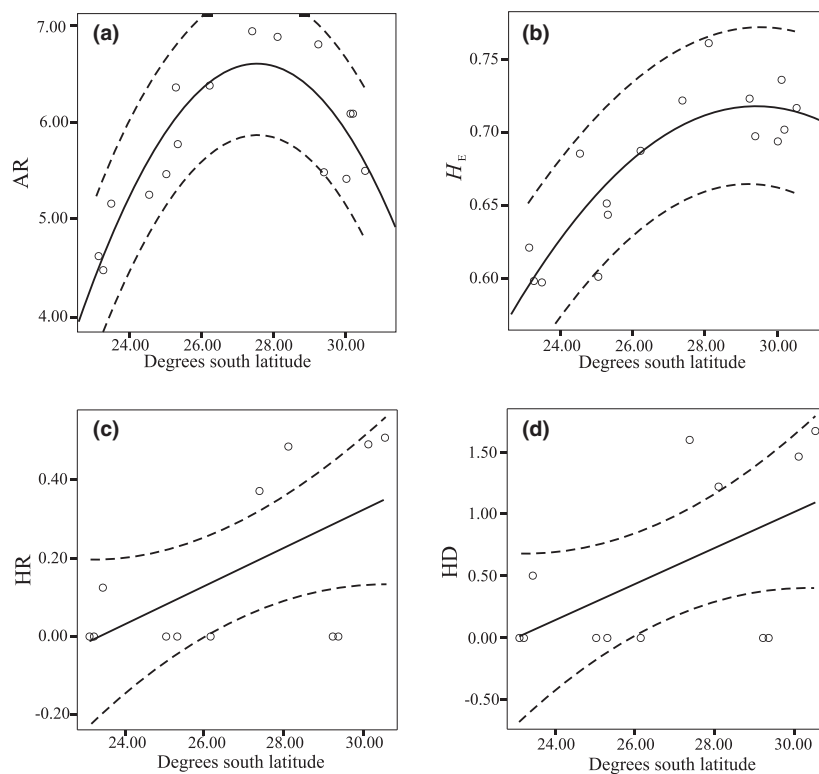


Figure 2 Geographical trends of genetic parameters of *Epidendrum fulgens* populations in southern and south-eastern Brazil. Correlation between latitude and (a) nuclear allelic richness (AR , $R^2 = 0.755$, $P < 0.05$) and (b) nuclear genetic diversity (H_E , $R^2 = 0.738$, $P < 0.0001$) described by the quadratic regression model, and (c) plastid haplotype richness (HR , $R^2 = 0.331$, $P < 0.05$) and (d) plastid haplotype diversity (HD , $R^2 = 0.239$, $P < 0.05$) described by the linear regression model, showing a significant decrease in diversity estimates towards the north. Dashed lines represent 95% confidence intervals calculated based on individual predicted y values from the regression line (as indicated in SPSS v.13.0).

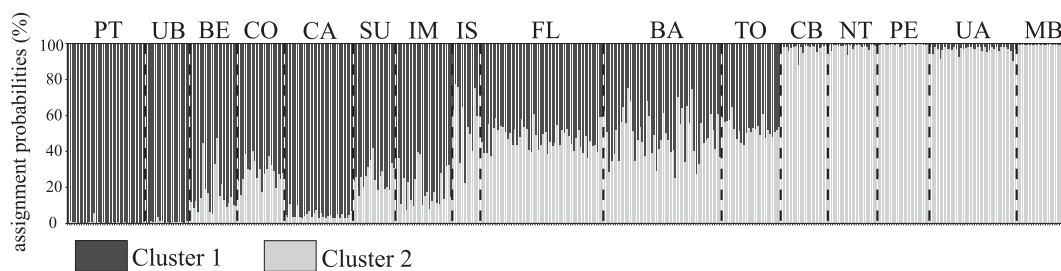


Figure 3 Summary of the population structure in *Epidendrum fulgens* in southern and south-eastern Brazil using Bayesian assignment analysis for a $K = 2$ population model. Cluster 1 corresponds to northern populations and cluster 2 to southern populations. See Table 1 for population identification.

Almost all individuals from the northernmost populations PT and UB show a strong assignment to cluster 1 ($Q < 0.95$), whereas the southernmost populations MB, UA, PE, NT and CB show admixture proportions strongly associated with cluster 2 ($Q > 0.95$). The remaining populations show intermediate admixture proportions between northern (PT and UB) and southern (MB, UA, PE, NT and CB) populations. AMOVA results indicate that the majority of the genetic variation resides within populations (91.64%, $P < 0.0001$), and only 8.36% of the nuclear diversity is found among populations.

Plastid DNA genetic structure

Statistical parsimony retrieved a well-resolved network (Fig. 1b), in which the most frequent and widespread haplotype (H1) was found in 69.6% of the individuals and in 10 out of 12 sampled populations. H1 is distributed from the northernmost population PT to population CB, which is situated on the southern border of the BAF (Fig. 1a). This was also the fixed haplotype in seven populations (PT, UB, CA, IM, IS, TO and CB). Haplotype H2 is restricted to the southern populations MB, PE and BA and forms the core of the network

topology, to which three other haplotypes are connected (H3, H4, H8). Haplotypes H5 and H7 are more differentiated and connected to the network by more than one mutational step. These divergent haplotypes are found in the Florianópolis and Imbituba populations, which also show higher levels of variation for nuclear and plastid markers. According to the AMOVA results, a high proportion of the genetic variability in the haplotype data resides among populations (60.57%, $P < 0.0001$), and only 39.43% ($P < 0.0001$) was attributed to within populations. Pairwise F_{ST} values among populations ranged from -0.050 to 0.859 (Table 3). In general, lower F_{ST} values were observed between adjacent populations (Table 3). Differentiation measures across all populations were high, with $F_{ST} = 0.605$ and $G_{ST} = 0.618$. A significant decrease in haplotype richness ($P < 0.05$) and haplotype diversity ($P < 0.05$) was found towards the north (Fig. 2c,d, respectively), similar to the pattern found for nuclear markers. For plastid markers, the relationship between genetic diversity and latitude was best described by a linear model (haplotype richness, $R^2 = 0.331$; haplotype diversity, $R^2 = 0.239$).

Historical population size reduction

Wilcoxon signed-rank tests did not reveal any significant excesses of heterozygosity under the TPM, indicating that

Table 4 Bottleneck test probabilities of 16 natural *Epidendrum fulgens* populations in southern and south-eastern Brazil based on estimates of nuclear multilocus genotypes. Wilcoxon signed-rank tests under the two-phase model of mutation (TPM) were used to test for heterozygote excess, and the mean Garza–Williamson index (M) was used to compare allele numbers with allele size ranges.

Population	TPM*	M †
Parati	0.981	0.646‡
Ubatuba	0.751	0.653‡
Bertioga	0.935	0.804
Ilha Comprida	0.849	0.815
Ilha do Cardoso	0.849	0.771
Ilha de Superagui	0.820	0.726
Ilha do Mel	0.751	0.770
Ilha de São Francisco	0.673	0.741
Florianópolis	0.820	0.834
Imbituba	0.500	0.878
Torres	0.714	0.743
Morro Santana	0.500	0.754
Morro São Pedro	0.367	0.777
Morro do Cabrito	0.975	0.777
Itapuã	0.986	0.794
Arambaré	0.632	0.740

*No values were significant ($P > 0.05$).

†A population is considered to have undergone a bottleneck if its M -value falls below a threshold of 0.680, following the procedure described by Garza & Williamson (2001).

‡Populations in which bottlenecks were detected according to Garza & Williamson (2001).

populations have not experienced a recent or a strong bottleneck. By contrast, M -ratios suggested that bottlenecks have occurred in the northern portion of the distribution of *E. fulgens*, in populations PT (0.646) and UB (0.653). Across all sites and loci, the M -ratio ranged from 0.646 to 0.878 (Table 4).

Pollen versus seed flow

Using the values of genetic differentiation G_{ST} among populations for nuclear (0.097) and plastid (0.618) markers, the ratio of pollen flow to seed flow was estimated at 14.25, suggesting that gene flow via pollen in *E. fulgens* is more than tenfold greater than that via seeds.

DISCUSSION

Deep disjunction between southern and northern populations

The most outstanding pattern recovered by the joint use of plastid and nuclear markers was the deep differentiation of populations of *E. fulgens* located south of Torres (seashore) and Morro do Cabrito (remnant calcareous outcrops from the lower slopes of the ‘Serra Geral’). This region represents the southern limits of the BAF (Fig. 1a), coincident with the end of the Serra Geral mountain chain. Located approximately between 29° and 30° S, this region of disjunction is recognized historically as an important phylogeographical boundary, called the ‘Portal de Torres’ (Rambo, 1950). The ‘Portal de Torres’ demarcates the northern limits of subtropical grassland plant communities and the southern limits of the BAF, and the deep genetic differentiation between populations within the BAF and those located on southern granitic outcrops probably reflects a historical vicariance pattern. Because the LGM had different impacts on forest and grassland plant communities, the results presented here highlight the importance of studies including species distributed across different biomes.

Genetic structure and diversity within the BAF

There is a lack of phylogeographical structure across the populations analysed within the BAF, considering both nuclear and plastid markers (Figs 1a & 3). In addition, nuclear markers show low genetic differentiation with regard to θ , G_{ST} and G'_{ST} , with the majority of the genetic variation being partitioned within populations. Indeed, there are no clear recognizable geographical barriers along the seashore where *E. fulgens* occurs, at least for populations from Parati to Torres. A different pattern has been reported for other species associated with humid forest (Cabanne *et al.*, 2007; Palma-Silva *et al.*, 2009; Ribeiro *et al.*, 2011), which show strong phylogeographical structure and evidence of past forest fragmentation, congruent with the contraction of the BAF during the LGM. With a decrease in temperature of $3\text{--}7^\circ\text{C}$ during the LGM, open scrub and grasslands were the dominant vegetation

in southern and south-eastern Brazilian lowlands close to the seashore, where tropical rain forest currently exists (Behling & Negrelle, 2001; Behling, 2002). As open scrub and grassland-type vegetation communities are the current habitats occupied by *E. fulgens*, the absence of phylogeographical structure in our results is in agreement with palaeovegetation reconstructions, suggesting a non-fragmented distribution during the LGM for this species. Our data are also in agreement with a scenario found for other non-forest species (Jakob *et al.*, 2009; Antonelli *et al.*, 2010; Cosacov *et al.*, 2010) and sand-dune plants (Kadereit *et al.*, 2005; King *et al.*, 2009), suggesting that during the LGM a significant proportion of species did not undergo dramatic range fragmentation into separate glacial refugia.

A marked decrease in genetic diversity was found in both nuclear and plastid markers towards the north (Fig. 2). The two northern populations PT and UB also showed signs of severe population decline (i.e. bottlenecks). The lower levels of genetic diversity coupled with the significant population size reduction detected in the northern distribution of *E. fulgens* suggest that the expansion of the BAF towards the south, after the LGM, played a role in limiting the availability of habitat for this species, reducing local densities. Formerly widespread species subjected to transitory reductions in population sizes, caused generally by bottlenecks or founder events, show a reduction of genetic diversity, a pattern observed in most phylogeographical analyses within the BAF biome (Cabanne *et al.*, 2007; Palma-Silva *et al.*, 2009; Ribeiro *et al.*, 2011). As a result of forest expansion and sea-level rise after the LGM, restinga vegetation was constrained to a narrow (20–50 m wide) and patchy distribution mainly between latitudes 23° and 24° S (Souza *et al.*, 2008), including the locations of populations PT, UB and BE. This pattern gradually changed towards the south, where sand dunes in restinga became wider (1–10 km) and more connected (Seeliger, 1992). When population isolation increases and population density decreases, genetic drift becomes stronger and reduces genetic diversity, mainly in peripheral populations (Eckert *et al.*, 2008). The occurrence of private alleles in populations in the northern part of the distribution of *E. fulgens* also indicates that individuals in those populations represent long-term persistence rather than recent migration from southern populations, as reported in the literature for a wide range of organisms (e.g. Burton, 1997; Stehlik *et al.*, 2002; Antunes *et al.*, 2006; Ortiz *et al.*, 2007; Jakob *et al.*, 2009).

Disjunct populations on granitic outcrops

A different picture is observed for southern populations of *E. fulgens*, where both diversity and structure were higher than in northern localities (Figs 1a & 2). Assignment probabilities show a tendency of differentiation for populations south of Torres, based on nuclear markers (Fig. 3). Plastid markers also show a marked differentiation in haplotype frequencies in the same region. The fixed haplotype (H1) found in Torres and Morro do Cabrito can be explained by an east–west migration

bridge of typical species from the BAF, from the seashore to the west over the lowlands of the Central Depression and the lower north-eastern slopes of the Serra Geral (Rambo, 1950; Leal & Lorscheitter, 2007). The Morro do Cabrito population shows nuclear admixture proportions characteristic of southern populations (Fig. 3) and haplotypes typical of northern populations (H1, Fig. 1a). This conflicting pattern found in Morro do Cabrito is probably due to its intermediate position, marking the border between the BAF and southern grassland biomes (Fig. 1a).

Populations of *E. fulgens* from southern granitic outcrops (populations NT, PE, UA) showed a considerable proportion of genetic diversity and a marked difference in allele frequencies for both nuclear and plastid markers. The remarkable genetic diversity of these populations suggests that these localities acted as refugial areas during oceanic transgressions. Successive marine transgressions occurring from the Late Pleistocene created a mosaic of swamps, lakes and small patches of forest in the lowlands, isolating the granitic outcrops from the seashore sand-dune vegetation (Seeliger, 1992) and constraining gene exchange with sand-dune populations located within the BAF. Floristic inventories of these outcrops reveal a considerable proportion of endemic and rare species (Ferreira *et al.*, 2010), indicating that these granitic hills were refugial areas during marine transgression events for both forest and grassland species (Rambo, 1954a). Both the higher elevation and the presence of patches of exposed rocks and shallow soil allowed the presence of grassland and open scrub plant communities even during warmer and more humid periods, creating the mosaic of forest/grassland species currently observed (Rambo, 1954a; Ferreira *et al.*, 2010). The Arambaré population was probably colonized from populations sheltered in the granitic hills, after Holocene sea transgressions, when sand dunes at this locality were formed (Seeliger, 1992).

Population genetic signatures and historical sea-level oscillation

The historical oscillation of sea level could also have played a role in limiting the plastid genetic diversity of populations from Parati to Torres. According to Seeliger (1992) and Behling (2002), successive marine transgressions and regressions occurred during the Pleistocene and Holocene, negatively affecting coastal vegetation as observed in the low number of endemic species in this habitat (Rambo, 1954b; Scarano, 2002). Generally, the restinga vegetation is composed of a subsample of species from the BAF and grasslands that are adapted to harsh environments and exhibit broad ecological amplitude (Scarano, 2002). Populations at Imbituba and Florianópolis are exceptions to this pattern, because high genetic diversity was observed for both nuclear and plastid markers. Those populations are directly associated with (Imbituba) or close to (Florianópolis) sheltered areas related to Pleistocene barriers (Hesp *et al.*, 2009). These barriers were sand deposits initiated

during the Last Interglacial, at *c.* 120 ka (Hesp *et al.*, 2009). The vegetation found on these barriers was protected during the sea-level oscillations of the late Holocene owing to their height above sea level (50–100 m). This offered a stable environment where sand-dune vegetation could persist, constituting a probable refugium for those species (Rambo, 1954b; Seeliger, 1992; Hesp *et al.*, 2009).

The role of gene flow in shaping broad-scale genetic patterns

Gene flow among *E. fulgens* populations proved to be tenfold greater via pollen than via seeds. This result is not expected for orchid species, which are characterized by dust-like seeds dispersed by wind (Arditti & Ghani, 2000). Moreover, nuclear markers did not show strong evidence of genetic structuring among *E. fulgens* populations, also suggesting high levels of gene flow by pollen. Gene flow between granitic outcrops and sand-dune populations within the BAF is probably constrained only by reduced seed dispersal, as the plastid genetic differentiation observed between adjacent populations is great (Table 3).

For two other food-deceptive orchids, *Galearis cyclochila* (as *Orchis cyclochila*, Chung *et al.*, 2005) and *Orchis purpurea* (Jacquemyn *et al.*, 2006), empirical data obtained for contemporary gene flow estimations suggest that seeds have only a limited dispersal capacity. In deceptive orchids outcrossing prevails because pollinators avoid plants in the same patch, promoting pollen gene flow over long distances and reducing the chances of geitonogamous pollination (Cozzolino & Widmer, 2005). Extensive pollen transport between *E. fulgens* populations is supported by the low inbreeding coefficients, high proportion of genetic diversity within populations and low population differentiation. The genetic signatures observed in populations of *E. fulgens* are in agreement with and confirm the genetic structure expected for food-deceptive orchids (Cozzolino & Widmer, 2005). The significant isolation-by-distance detected and the low differentiation between adjacent populations (Table 3) suggest that pollen dispersal performed by butterflies may be restricted to neighbouring populations. However, the lack of nuclear genetic structure among populations may suggest pollen transport by butterflies over longer distances. As reviewed by van der Cingel (2007), species of Lepidoptera can contribute substantially to pollen transport of orchid species over long distances. Results found for other food-deceptive orchids showed different patterns, however. For *Anacamptis palustris* (Cozzolino *et al.*, 2003) and *Dactylorhiza majalis* (Nordström & Hedrén, 2009), seed-mediated gene flow was greater than gene flow via pollen (pollen:seed flow ratios of 0.48 and 0.30, respectively). According to the authors, these results need to be interpreted with caution as populations of those species are restricted to specialized habitats and show a disjunct, patchy distribution, which constrains gene flow among populations (Cozzolino *et al.*, 2003; Nordström & Hedrén, 2009). Fine-scale spatial genetic structure coupled with progeny analyses (Chung *et al.*,

2005; Jacquemyn *et al.*, 2006) may clarify the role of pollen-mediated gene flow in *Epidendrum* populations.

Populations on range margins displayed lower genetic diversity than did central populations, mainly for nuclear allelic richness (Fig. 2a). Increased drift resulting from reduced gene flow on marginal populations (Table 3), combined with reduced density (Allee effect) and reduced mutational input in marginal populations, may contribute to the reduced levels of diversity observed at range margins (Bridle & Vines, 2007; Eckert *et al.*, 2008). Theoretical models of evolution in range margins commonly consider factors such as density, drift, gene flow and selection along a *selective gradient* (Kirkpatrick & Barton, 1997; Bridle & Vines, 2007), that is, at a spatial scale that is finer than the one studied here; future studies on the limits of evolution in the range margins of *E. fulgens* should explicitly focus on this scale at both the northern and southern margins.

CONCLUSIONS

The present phylogeographical study of *E. fulgens* reveals the influence of contrasting and complex geological and climatic events on patterns of diversification and distribution in this orchid species. The deep disjunction detected between northern populations within the BAF region and southern populations outside the BAF is probably associated with successive marine transgressions occurring from the Late Pleistocene. This historical vicariance pattern is geographically coincident with the northern limits of subtropical grassland plant communities and the southern limits of the BAF, traditionally called 'Portal de Torres'. The scenario depicted by our analytical approach suggests that *E. fulgens* was widespread during the LGM, rather than being isolated in only a few refugia. The expansion of grassland habitats, as revealed by palynological data for the LGM, apparently had a positive effect on the population persistence of species associated with sand-dune and rock outcrop plant communities. Populations found on Pleistocene sand barriers and granitic outcrops in the southern part of the range of *E. fulgens* showed increased levels of genetic diversity, probably due to the age and stability of those locations. Bottlenecks were detected in the two northern populations, suggesting that forest expansion towards the south after the LGM, followed by narrowing of the coastal sands and sea-level rise, played a role in the population fragmentation and decrease in genetic diversity in this species. Private alleles found across most of the populations indicate the long-term persistence of these populations, rather than recent migrations. The phylogeographical pattern for this coastal orchid species differs substantially from that of forest-associated species within the BAF, which underwent substantial bottlenecks in glacial refugia during the Pleistocene (Cabanne *et al.*, 2007; Palma-Silva *et al.*, 2009; Ribeiro *et al.*, 2011). The results highlight the substantial contribution of species growing outside the forests to our knowledge of past vegetation and climate dynamics in the Neotropics.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Overview of the main types of habitats where *Epidendrum fulgens* can be found.

Appendix S2 *Epidendrum fulgens* natural growing conditions and flower morphology.

Appendix S3 Magnitude of ΔK from STRUCTURE analysis as a function of *K*.