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Reproductive barriers and genetic differentiation between continental and island populations of *Epidendrum fulgens* (Orchidaceae)

Fábio Pinheiro[1] · Giovanna Seleghin Veiga[1] · Cleber Juliano Neves Chaves[1] · Tami da Costa Cacossi[1] · Clarisse Palma da Silva[1]

Abstract

Studies on insular organisms provide an important framework for investigating patterns of genetic differentiation and reproductive isolation. By focusing on populations of the same species, we have the opportunity to study 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 genetic differentiation and the evolution of early stages of intrinsic postmating reproductive isolation between continental and insular populations of *Epidendrum fulgens*, a neotropical orchid distributed in southeastern Brazil. Genetic diversity and structure were estimated for both nuclear and plastid markers by using genetic differentiation measures and model-based assignment test. Furthermore, two components of reproductive isolation were examined by analyzing fruit set and seed viability in interpopulation crosses. Strong plastid genetic structure ($F_{ST} = 0.679$) was found between insular and remaining populations, indicating that *E. fulgens* reduced gene flow via seed dispersal, although significant nuclear genetic structure was lower ($D_{ST} = 0.179$), likely due to the smaller effective population size of the plastid genome. Significant differences in seed viability between self- and cross-pollinated plants were found, indicating the occurrence of inbreeding depression in all populations. Seed viability was significantly lower in crosses between insular and remaining populations, suggesting a late postzygotic reproductive barrier due to low migration associated with genetic drift (i.e., bottleneck) and its consequences. Our results confirm the importance of islands as drivers for populational differentiation and suggest a central role for outbreeding depression during the early stages of lineage diversification.

Keywords Genetic drift · Gene flow · Inbreeding depression · Outbreeding depression · Reproductive isolation · Speciation

Introduction

The spatial isolation of populations may restrict connectivity, leading to low levels of gene flow between patches, with subsequently higher genetic differentiation and lower genetic diversity in insular populations (Ellstrand 2014). Such differentiation is often attributed to the low levels of gene exchange and drift, as discussed by previous studies focused on plants with naturally fragmented ranges (Palma-Silva et al. 2011; Pinheiro et al. 2014). Indeed, the high levels of endemism found in oceanic islands and rock outcrops highlight the role of gene exchange reduction and drift in speciation events (Porembski & Barthlott 2000; Ballesteros-Mejia et al. 2016; Cabral et al. 2019). If island endemics are a product of the low levels of gene exchange between island and mainland species, we may expect similar levels of gene exchange reduction between populations of the same species occurring in both insular and continental habitats. In

Handing editor: Mario Fernández-Mazuecos.

✉ Fábio Pinheiro
   biopinheiro@yahoo.com.br

   Giovanna Seleghin Veiga
   giovanna.slvg@gmail.com

   Cleber Juliano Neves Chaves
   cleberchaves@gmail.com

   Tami da Costa Cacossi
   tamiccacossi@gmail.com

   Clarisse Palma da Silva
   clarissepalma@yahoo.com.br

[1] Departamento de Biologia Vegetal, Instituto de Biologia, Universidade Estadual de Campinas, Campinas, SP, Brazil

this scenario, the reduction of gene exchange between island and mainland populations can be followed by an increase in reproductive isolation barriers. Reproductive experiments focused on different populations within species are crucial to understand how reproductive barriers arise within species, clarifying the first steps of speciation (Via 2009). Studies estimating diversification (Nürk et al. 2019) and evolution of reproductive barriers (Crawford and Stuessy 1997) usually compare between insular plants and their non-insular relatives, thus reducing our ability to distinguish microevolutionary factors at the beginning of speciation from those accumulating subsequent to divergence (Crawford and Archibald 2017).

The ability to self-fertilize may provide reproductive assurance during island colonization (Baker 1955; Herlihy and Eckert, 2002; Leimu 2004), decreasing the extinction risk of selfing species when compared with outcrossers (Lennartsson 2002). Changes in reproductive traits have been widely documented in plants (Barrett 2002), suggesting that mating system may respond to various ecological scenarios (Pannell et al. 2015). However, transitions to selfing may lead to an increase in the expression of recessive deleterious alleles, which may compromise the colonization of new habitats due to inbreeding depression. Thus, the purging of recessive deleterious alleles may decrease the effects of genetic load and inbreeding depression (Charlesworth and Charlesworth 1987) facilitating the colonization of new habitats by plants relying on self-pollination (Barrett 1996).

Several scattered islands are found in the southeastern Brazilian coast, distributed over a broad 10–40 m deep coastal shelf. Most islands were connected to the mainland during the last glacial period (115,000–10,000 years ago), where sea level was up to 110 m below the current level (Suguio et al. 1988). Several minor transgressions and regressions also occurred in the last 10,000 years, changing the degree of isolation and the area of many islands and the neighboring coastal plain (Suguio et al. 1985). These historical sea level changes may have played an important role in shaping the evolution of different organisms in this region (Olmos 1996; Leite et al. 2016), including the evolution of insular endemics. Alcatrazes Island (Fig. 1a), with 2.5 km length and a total area of 0.68 km², is one of the most isolated coastal islands in southeastern Brazil, distant from the continent by 35 km. Despite most of the island's flora and fauna being shared with the neighboring coastal region (Pompéia et al. 1993), some endemic plants (Hoehne 1958; Coelho and Catharino 2008) and animals (Marques et al. 2002; Brasileiro et al. 2007) have been reported for Alcatrazes Island. However, studies aiming to investigate the genetic differentiation and reproductive isolation of mainland and island populations were rarely conducted in the region (but see Rocha Filho et al. 2013). Due to its relative geographic isolation, we hypothesize that species occurring in both mainland and Alcatrazes Island may show genetic differences and reproductive isolation due to restrictions in dispersal and consequentially limited gene exchange.

In this study, we used the coastal orchid species *Epidendrum fulgens* (Fig. 1b) as a model to investigate how insular isolation may impact the genetic variation and reproductive isolation of populations. This orchid is distributed along the

Fig. 1 General view of Alcatrazes Island (**a**), where samples of *Epidendrum fulgens* were collected in vegetation clumps fixed along granitic slopes (**b**), where vegetative propagation may provide an advantage in this harsh environment (**c**). Photograph credits: **a** Elsie Rotenberg, **b**, **c** Fábio Pinheiro
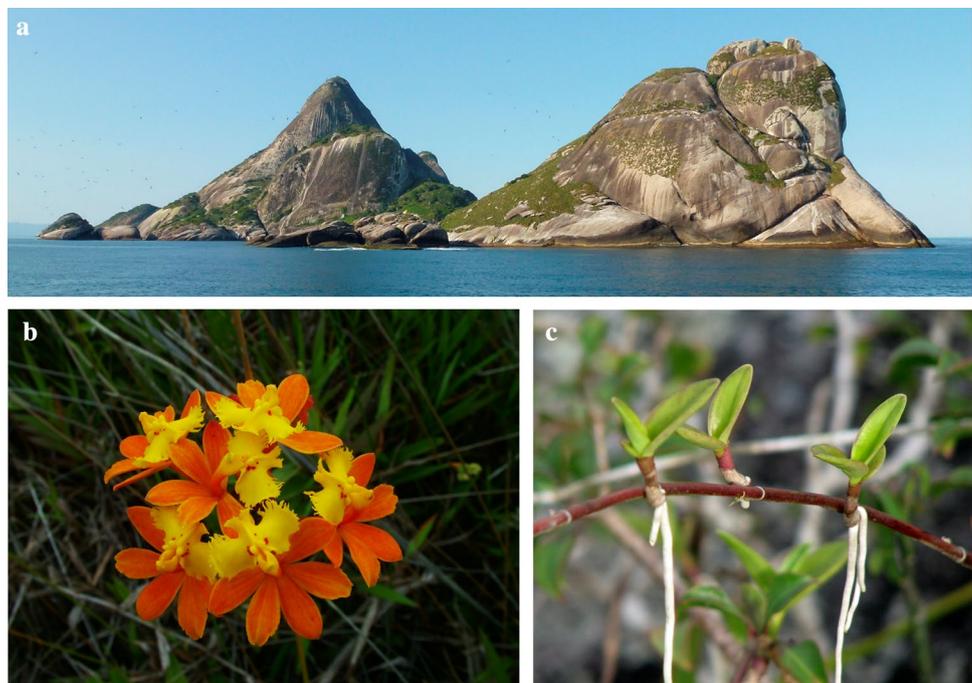
south and southeastern Brazilian coast (Sujii et al. 2019), between Rio Grande do Sul and Rio de Janeiro States. This species is self-compatible, but fruit set is dependent on pollinator visits (Fuhro et al. 2010). Indeed, *E. fulgens* shows high levels of outcrossing (Sujii et al. 2019), being pollinated by different butterfly species (Fuhro et al. 2010) and having seeds dispersed by wind. *Epidendrum fulgens* is diploid ($2n = 24$) and shows regular meiosis (Moraes et al. 2013; Pinheiro et al. 2015). The species is very common in coastal sand dune vegetation, in which thousands of plants are easily found (Pinheiro et al. 2011; Sujii et al. 2019). Our previous data showed low genetic differentiation for both nuclear and plastid markers among populations occurring along the coastal vegetation (Pinheiro et al. 2011). Here, we aim to answer the following questions: (a) Is there genetic differentiation between Alcatrazes Island and remaining populations? (b) Is there evidence of inbreeding depression in Alcatrazes Island, when compared with continental populations? (c) Does reproductive isolation occur between Alcatrazes Island and the remaining populations? Here, we found strong genetic differentiation between continental and Alcatrazes Island populations of *Epidendrum fulgens*, mainly at plastid markers. Genetic differentiation was associated with a significant decrease in two components of reproductive isolation, fruit set and seed viability. Inbreeding depression was detected in both continental and insular populations, suggesting deleterious alleles may challenge the persistence of the species in a small and restricted insular environment.

Material and methods

Plant sampling

Five populations were sampled for crossing experiments: four continental populations occurring in coastal sand

vegetation, and one population from Alcatrazes Island (Table 1, Fig. 2), which is 35 km apart from the southeastern Brazilian coast. *Epidendrum fulgens* is found on coastal vegetation growing in sand dunes in most of its continental distribution. In contrast, at Alcatrazes Island, *E. fulgens* grows in vegetation clumps distributed in granitic rock slopes. Due to the difficulty in reaching the places where the plants grow, only nine plants were collected. We sampled plants at a minimum distance of 5 m, distributed in different vegetation clumps, which are composed predominantly of species of Bromeliaceae, Cactaceae and Araceae (Pompéia et al. 1993). Reproductive experiments were performed in plants kept in cultivation from all populations (Table 2). For genetic analysis, we used samples collected at Alcatrazes Island, genotyped for the first time in this study, and continental samples previously genotyped by Pinheiro et al. (2011) and Sujii et al. (2019), totalizing 18 populations (see Fig. 1 and Online Resource 1 for further details).

Crossing experiments

Crossing experiments were conducted according to Pinheiro et al. (2013). The experimental treatments included untouched flowers, emasculation, manual self-pollination, cross-pollinations within populations, and cross-pollinations using plants from different populations. Crosses were performed bi-directionally, with each flower providing and receiving pollen from the intrapopulation or interpopulation partner, respectively. In total, 197 flowers from 26 plants were crossed. Fruit development was monitored until the fruits were mature (as evidenced by opening of ripe fruits). Fruit set was measured by dividing the number of mature fruits by the total number of pollinated flowers. Then, mature fruits were collected and checked for the presence of seeds, which were immersed in a 1% solution of 2,3,5- triphenyl tetrazolium and stored for 24 h at 30 °C. Following this procedure, viable embryos were stained a

Table 1 Genetic diversity results obtained for *Epidendrum fulgens*, using nine nuclear and six plastid microsatellite markers, including the number of individuals analyzed for nuclear (N_n) and plastid (N_p) markers, number of alleles (A), allelic richness (AR), the expected (H_E) and observed (H_O) heterozygosities, the within population inbreeding coefficient G_{IS} for nuclear microsatellites, the number of plastid DNA haplotypes found (*NH*), the haplotype diversity (HD) and haplotype richness (HR)

Population	Nuclear microsatellites						Plastid microsatellites			
	N_n	A	AR	H_E	H_O	G_{IS}	N_p	*NH*	HD	HR
Alcatrazes Island	9	35	3.78	0.530	0.622	− 0.174*	9	2	1.000	0.389
Ubatuba[1]	18	49	4.49	0.599	0.574	0.042	8	1	0.000	0.000
Bertioga[1]	20	63	5.17	0.597	0.454	0.245*	16	2	0.500	0.125
Comprida[1]	20	77	5.26	0.686	0.616	0.105*	16	1	0.000	0.000
Cardoso[1]	29	76	5.48	0.601	0.580	0.036	16	1	0.000	0.000

[1]Data from Pinheiro et al. (2011); *Departures of within-population inbreeding coefficients (*f*) from the Hardy–Weinberg equilibrium (HWE) are indicated by asterisks ($P < 0.01$)
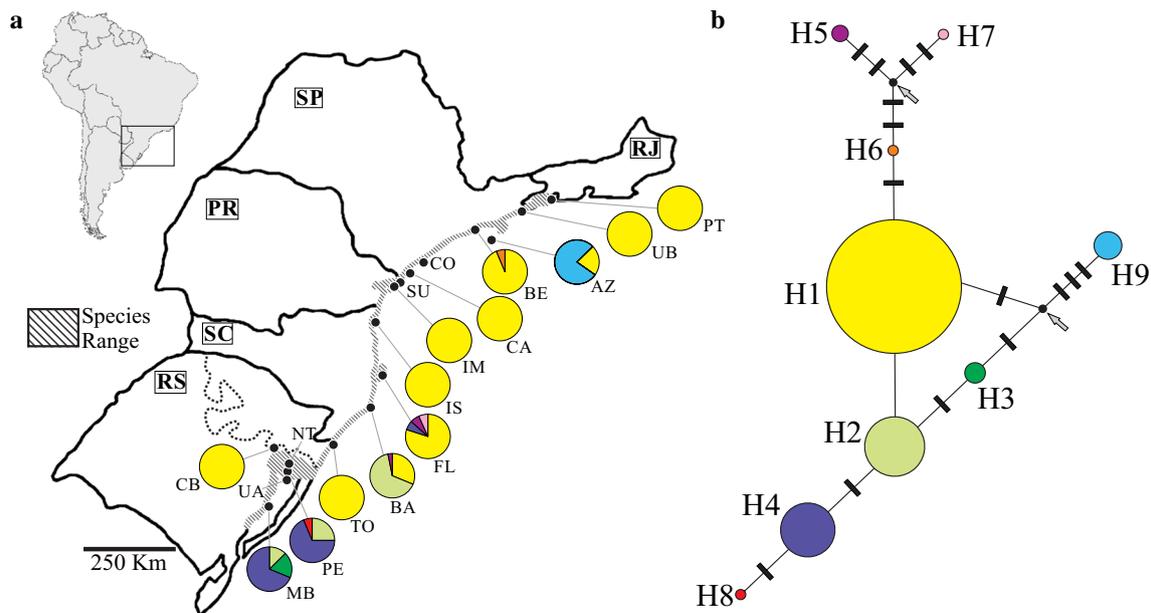
Fig. 2 Map showing sampled populations of *Epidendrum fulgens* along the south and southeastern Brazilian coast, including the Alcatrazes Island (**a**) and the plastid DNA network (**b**). Pie charts represent the frequency of occurrence of haplotypes in each population. Haplotype colors correspond to those shown in networks. In the statistical parsimony network (**b**), the haplotype frequencies are proportional to circle sizes. The number of mutations required to explain transitions among haplotypes is indicated along the lines connecting the haplotypes by crosshatches. The arrows indicate missing inter-

mediate haplotypes not found in the analyzed individuals. Brazilian Federal States: Rio Grande do Sul (RS), Santa Catarina (SC), Paraná (PR), São Paulo (SP), Rio de Janeiro (RJ). Population codes are as follows: *PT* Paraty, *UB* Ubatuba, *AZ* Alcatrazes Island, *BE* Bertioga, *CO* Comprida, *CA* Cardoso, *SU* Superagui, *IM* Ilha do Mel, *IS* Ilha de São Francisco, *FL* Florianópolis, *BA* Imbituba, *TO* Torres, *NT* Morro Santana, *PE* Morro São Pedro, *CB* Morro do Cabrito, *UA* Itapuã, *MB* Arambaré. Modified from Pinheiro et al. (2011)

strong red color. At least 200 seeds from each fruit were analyzed under microscope. The percentage of viable seeds was calculated by dividing the number of viable embryos by the total number of embryos scored. Fruit and seed data were obtained for each cross-type and compared constructing general linear mixed-effects models (GLMMs) followed by chi-squared analysis, with *lme4* R package (Bates et al. 2015), using the binomial data of seed viability as fixed-effect and paternal (i.e., the pollen donor) and maternal (i.e., the pollen receptor) genotypes as a random effect. Pairwise comparisons among populations were performed through a Tukey test, with *multcomp* R package (Hothorn et al. 2008).

Genetic analysis

Continental samples were previously genotyped by Pinheiro et al. (2011) and Sujii et al. (2019) using nine nuclear SSR (nuSSR) loci (eff26, eff29, eff43, eff45, eff61, eff70, epp10, epp18, epp86; Pinheiro et al. 2008a, 2008b) and six plastid SSR (cpSSR) loci (EPCP02, EPCP03, EPCP04, EPCP06, EPCP08, EPCP09; Pinheiro et al. 2009). Samples from Alcatrazes Island were genotyped for the first time, using the same loci. For each individual plant collected, fresh leaves were dried in silica gel and stored at room temperature until

laboratory analysis. Sample sizes are shown in Table 1. Genotyping assays followed Pinheiro et al. (2011). 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 with Micro-Checker (van Oosterhout et al. 2004).

Genetic diversity and structure parameters were calculated for Alcatrazes Island and four adjacent continental populations, Ubatuba, Bertioga, Comprida and Cardoso (Table 1). The nuSSR diversity of each population was characterized using the number of alleles (A) and allelic richness (AR), and the inbreeding coefficient G_{IS}, calculated using the Genodive program (Meirmans and Van Tienderen 2004). Genetic differentiation of nuSSR was measured using F_{ST}, which is an estimate largely used in population genetics, enabling comparisons among different studies, and D_{ST} (Jost 2008), which is independent of the levels of diversity found within populations, giving a better estimate of population differentiation.

The cpSSR diversity was estimated for Alcatrazes Island and continental populations Ubatuba, Bertioga, Comprida and Cardoso, using the number of haplotypes and haplotype richness calculated by the program rarefac 3.5 (Petit et al. 1998). The genetic differentiation of cpSSR was measured

Table 2 Fruit formation and viable seeds of *Epidendrum fulgens*, produced from self and different cross-pollination treatments, including the number of plants used as seed parents and pollen donors (*N*), the number of pollinated flowers (Flower), number of fruits produced (Fruit), the ratio between fruits produced by pollinated flowers (FR/FL), and seed viability (SV)

Pollen receptor (N) Bagged flowers	Pollen donor (N)	N^1	Flower	Fruit	FR/FL (SD)^2	SV (SD)^3
Alcatrazes Island (5)	–	5	10	0	–	–
Ubatuba (5)	–	5	10	0	–	–
Bertioga (5)	–	5	10	0	–	–
Cardoso (5)	–	5	10	0	–	–
Total emasculation		20	40	0	–	–
Alcatrazes Island (5)	–	5	10	0	–	–
Ubatuba (5)	–	5	10	0	–	–
Bertioga (5)	–	5	10	0	–	–
Cardoso (5)	–	5	10	0	–	–
Total self-pollinations		20	40	0	–	–
Alcatrazes Island (6)	–	6	14	14	1.00 (0.00)	0.23 (0.24)a
Ubatuba (5)	–	5	8	8	1.00 (0.00)	0.29 (0.13)a
Bertioga (5)	–	5	6	6	1.00 (0.00)	0.34 (0.09)a
Comprida (5)	–	5	7	7	1.00 (0.00)	0.42 (0.08)a
Cardoso (5)	–	5	7	7	1.00 (0.00)	0.49 (0.07)a
Total Continent × Continent crosses		26	42	42	1.00 (0.00)	0.33 (0.18)*
Alcatrazes Island (6)	–	6	16	16	1.00 (0.00)	0.48 (0.26)a
Bertioga (5)	–	5	16	14	0.87 (0.34)	0.80 (0.11)b
Comprida (2)	–	2	4	4	1.00 (0.00)	0.71 (0.12)ab
Cardoso (7)	–	7	17	16	0.94 (0.24)	0.71 (0.18)b
Total Continent × Island crosses		20	53	50	0.94 (0.23)	0.66 (0.23)*
Cardoso (2)	Ubatuba (2)	4	5	5	1.00 (0.00)	0.79 (0.07)
Ubatuba (2)	Cardoso (2)		4	3	0.75 (0.50)	0.75 (0.13)
Comprida (2)	Cardoso (4)	6	5	5	1.00 (0.00)	0.64 (0.15)
Cardoso (4)	Comprida (2)		6	5	0.83 (0.40)	0.63 (0.20)
Bertioga (1)	Comprida (1)	2	2	2	1.00 (0.00)	0.72 (0.07)
Comprida (1)	Bertioga (1)		2	1	0.50 (0.70)	0.74 (0.00)
Bertioga (1)	Ubatuba (1)	2	2	2	1.00 (0.00)	0.70 (0.05)
Ubatuba (1)	Bertioga (1)		2	2	1.00 (0.00)	0.82 (0.03)
Total Continent × Island crosses		14	28	25	0.89 (0.31)	0.71 (0.13)**
Alcatrazes I.(5)	Cardoso (3)	8	15	13	0.87 (0.35)	0.44 (0.29)
Cardoso (3)	Alcatrazes I.(5)		18	15	0.83 (0.38)	0.51 (0.28)
Alcatrazes I.(2)	Comprida (2)	4	4	4	1.00 (0.00)	0.04 (0.06)
Comprida (2)	Alcatrazes I.(2)		4	4	1.00 (0.00)	0.12 (0.01)
Alcatrazes I.(2)	Bertioga (2)	4	4	4	1.00 (0.00)	0.58 (0.34)
Bertioga (2)	Alcatrazes I.(2)		4	3	0.75 (0.50)	0.18 (0.24)
Alcatrazes I.(2)	Ubatuba (2)	4	4	2	1.00 (0.00)	0.37 (0.27)
Ubatuba (2)	Alcatrazes I.(2)		4	3	0.75 (0.50)	0.54 (0.36)
Total		20	57	48	0.82 (0.38)	0.45 (0.28)**

[1]Total number of plants used. [2]All comparisons were not significantly different ($P > 0.05$). [3]The same letter indicates that the means are not significantly different ($P > 0.05$). *Significant difference between treatments (self-pollinations and intrapopulation crosses; $P < 0.0001$). **Significant differences between continent vs. continent and continent vs. island crosses; $P < 0.01$)

using F_{ST}, with the Genodive program. The evolutionary history of plastid haplotypes was investigated using a median-joining network (Bandelt et al. 1999) using the program NETWORK v. 10.2.0.0 (www.fluxus-engineering.com). The haplotype network was built using samples from 13 populations and 200 individuals, including nine samples from Alcatrazes Island and the remaining previously genotyped by Pinheiro et al. (2011).

In order to quantify the number of clonal plants occurring in Alcatrazes Island, we calculated the ratio between the

number of genotypes and the total number of individuals in the population (G/N), where values close to zero indicate strict clonality, while values equal to one indicate sexual reproduction. Individuals with the same genotype can be either ramets of the same genet or derive by chance from distinct events of sexual reproduction. In order to explore the origin of plants with the same genotypes, we used the GenAlEx 6.5 program (Peakall and Smouse 2012) to estimate the probability that two individuals, randomly sampled from a population, share the same genotype by chance (probability of identity: PI). These analyses were performed only in Alcatrazes Island plants because Pinheiro et al. (2011) did not find any clonal plants in the remaining populations.

To explore the presence of distinct genetic clusters and patterns of admixture between populations, we used the Bayesian clustering approach as implemented in MavericK v. 1.0.4. (Verity and Nichols 2016), which uses thermodynamic integration to estimate the correct number of clusters present in the dataset. According to Verity and Nichols (2016), Maverick outperforms other methods in detecting genetic groups, including the ability to find support for just one genetic cluster ($K = 1$). We tested K from 1 to 5 with a burn-in of 200 000 steps followed by 10,00,000 Markov chain Monte Carlo iterations with 10 replicates to confirm stabilization of the summary statistics. Estimates were carried out under the admixture model. In this analysis, we included samples from Alcatrazes Island and all other continental samples previously genotyped by Pinheiro et al. (2011) and Sujii et al. (2019), totalizing 18 populations and 418 individuals (see Online Resource 1 for further details).

Results

Fruit set and seed viability

Changes in reproductive traits were not observed among populations, as bagged and emasculated flowers did not produce any fruit (Table 2). On the other hand, most hand pollinations produced fruits (Online Resource 2), and no significant difference ($P > 0.05$) was found in fruit set comparisons between different treatments described in Table 2. Mean seed viability values observed across populations ranged from 23.0 to 49.0% in self-pollinations and were significantly lower than values observed in cross-pollinations ($\chi^2 = 23.326$, $P < 0.001$), which ranged from 48.0 to 80.0% (Fig. 3a, Table 2). We observed no differences related to self-pollination results among populations ($\chi^2 = 1.613$, $P > 0.05$), but flowers subjected to intrapopulation crosses within Alcatrazes Island showed significantly less seed viability than flowers from Bertioga and Cardoso populations ($\chi^2 = 11.383$, $P > 0.01$; Table 2). Similar mean seed viabilities were observed when considering only crosses among continental

populations, ($\chi^2 = 0.1536$, $P = 0.9847$), with values ranging from 63 to 82% (Table 2). However, a significant decrease in seed viability ($\chi^2 = 8.899$, $P < 0.01$) was found when comparing mean values from crosses among continental populations ($N = 25$, 0.71 ± 0.13 SD) and those between continental populations and Alcatrazes island ($N = 43$, 0.45 ± 0.28 SD, Fig. 3b, Table 2). The decrease in seed viability detected in crosses between continental and Alcatrazes Island populations was not associated with any asymmetries when island plants acted as pollen donors ($N = 22$, 0.47 ± 0.28) or seed parents ($N = 21$, 0.43 ± 0.28).

Genetic diversity and structure

In general, genetic diversity estimates based on nuclear markers show lower values at Alcatrazes Island (Table 1). Even considering allelic richness values, where the sample size effect was removed (rarefaction method), continental populations show higher values when compared with those from Alcatrazes Island. The inbreeding coefficient G_{IS} was significantly different from zero in Bertioga and Comprida populations, and lower values were observed for the remaining populations. A significant negative G_{IS} value was found for Alcatrazes Island.

The analysis of six plastid loci recovered a total of nine haplotypes for *E. fulgens* (Fig. 1, Table 1, Online Resource 3). Two haplotypes were found in Alcatrazes Island, increasing diversity parameters estimates when compared with values observed in continental populations (Table 1). Most plants from the island had an exclusive haplotype (H9), detected here for the first time (Fig. 1). The other haplotype (H1) is also found in most continental populations. The new haplotype found at Alcatrazes Island is differentiated by several mutational steps from haplotypes found in continental populations (Fig. 1). The origin of haplotype H9 is ambiguous since it is connected to both haplotypes H1 and H3, separated by the same number of mutational steps (Fig. 1).

Clonal plants were found in Alcatrazes Island, in which four different genotypes were identified from nine genotyped plants (G/N ratio = 0.44). Seven out of nine plants correspond to only two nuclear genotypes, in which the exclusive island plastid haplotype was also found. In addition, a very low value of probability of identity (PI) was found for Alcatrazes Island plants (2.1×10^{-5}). Using Micro-Checker software (van Oosterhout et al., 2004), we found no evidence for scoring error due to "stuttering" or "large allele dropout."

Significant levels of genetic differentiation among populations ($P < 0.001$) were found for nuclear D_{ST} (0.179) and plastid F_{ST} (0.679). All pairwise nuclear D_{ST} values were significant, ranging from 0.019 to 0.357 (Table 3). Pairwise plastid F_{ST} ranged from 0.000 to 0.814, and significant values were found only in comparisons between Alcatrazes Island and remaining populations (Table 3). Pairwise

Fig. 3 Seed viability produced by hand pollinations of *Epidendrum fulgens*, including self-pollinations (orange) and cross-pollinations (green) within populations (**a**), and cross-pollinations among continental populations, between continental and Alcatrazes Island populations pooled, and when Alcatrazes Island acted as pollen donor and receptor separately (**b**). Asterisks represent outliers, diamonds are mean values, and horizontal lines inside boxplots are medians. The same lowercase letters indicate that the means are not significantly different ($P > 0.05$) according to statistical tests
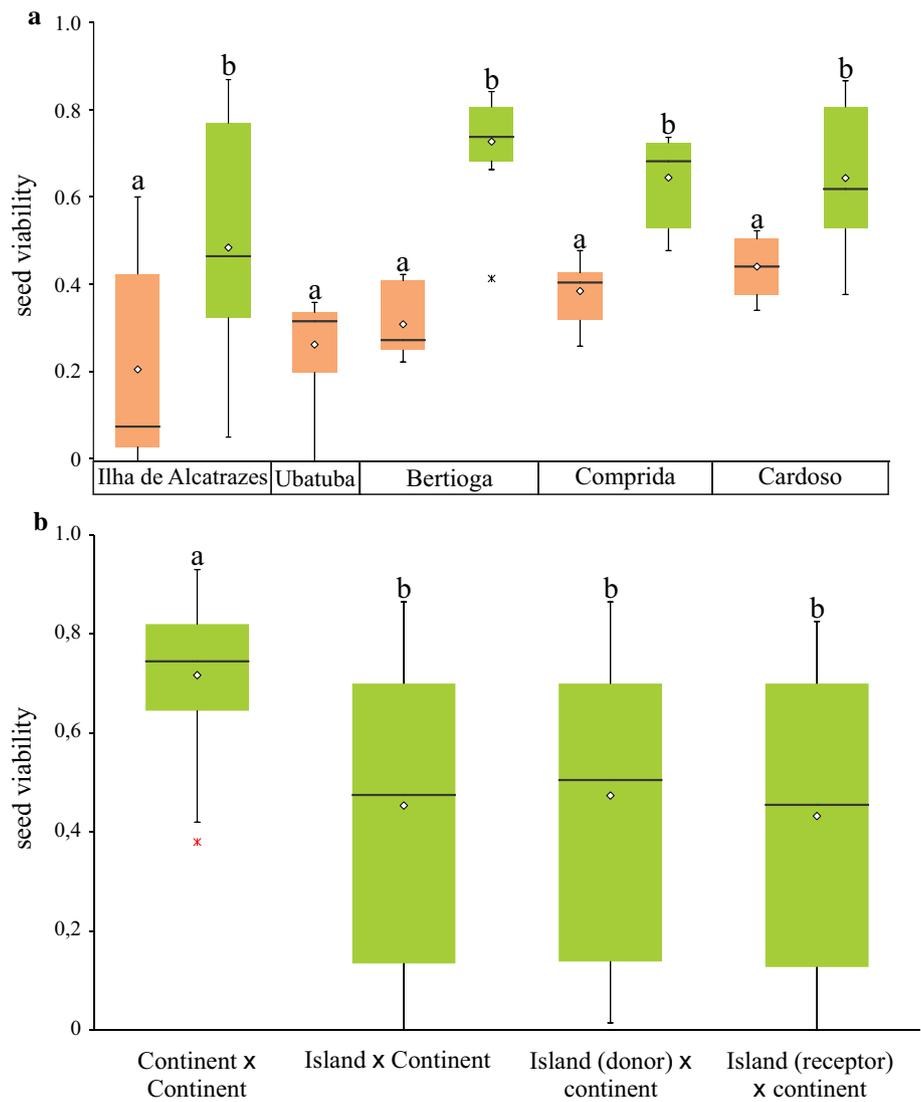
Table 3 Pairwise comparisons between populations of *Epidendrum fulgens* based on D_{ST} calculated on nuclear SSRs (below diagonal) and F_{ST} calculated on plastid SSRs (above diagonal)

	Alcatrazes Island	Ubatuba	Bertioga	Comprida	Cardoso
Alcatrazes Island	*	**0.736**	**0.714**	**0.814**	**0.814**
Ubatuba	**0.357**	*	− 0.050	0.000	0.000
Bertioga	**0.214**	**0.129**	*	0.000	0.000
Comprida	**0.107**	**0.256**	**0.176**	*	0.000
Cardoso	**0.087**	**0.259**	**0.177**	**0.019**	*

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

nuclear F_{ST} were all significant, ranging from 0.011 to 0.209 (Table 3).

Simulations performed in MaveriK consistently identified $K = 2$ clusters (Online Resource 1). Despite the identification of two genetic clusters, admixed individuals are present in most populations. The frequencies of each genetic cluster show some differences among populations.

Cluster 1 (dark gray) shows higher frequencies in Alcatrazes Island and in the remaining continental populations distributed in the Northern portion of *E. fulgens* geographic range (Online Resource 1, Fig. 2). Cluster two (light gray) is predominant in southern *E. fulgens* populations (Online Resource 1).

Discussion

Contrasting patterns of genetic differentiation were found for nuclear and plastid markers (Figs. 2, Table 3). Although genetic differentiation was significant among most populations (Table 3), low values were found for nuclear markers. In fact, nuclear admixture occurs among all populations (Online Resource 1). On the other hand, one private haplotype was found in Alcatrazes Island, indicating strong genetic differentiation with the remaining populations (Fig. 2, Table 3). This pattern mirrors the findings of Pinheiro et al. (2011), where genetic differentiation among *E. fulgens* populations was higher for plastid than nuclear markers. Drift may have a strong impact on the plastid genome due to its lower effective population size, accelerating the accumulation and fixation of new haplotypes. This pattern is also common for most Angiosperms (Petit et al. 2005) and is also related to the ratio of pollen to seed dispersal. Overall, low levels of seed dispersal are observed in flowering plants, when compared with pollen, increasing the genetic structure observed in plastid markers (Petit et al. 2005). However, two individuals in Alcatrazes Island were found carrying a haplotype also found in continental populations (Fig. 2), suggesting the persistence of this haplotype from the time the island was connected to the continent and colonized by *E. fulgens*, or a more recent and occasional long-distance dispersal event mediated by seeds. Unfortunately, we are not able to distinguish both scenarios. In the case of occasional long-distance dispersal between continent and islands, such events are expected to alleviate the effects of severe bottlenecks occurring during island colonization as reported for some plants (Barrett 1996). Thus, in *Epidendrum fulgens*, a non-endemic species from Alcatrazes Island, the rare events of long distance seed dispersal may contribute to balance the negative effects of drift and genetic load, contributing to the resilience of the species within this island.

Nuclear genetic diversity was slightly lower in Alcatrazes Island (Table 1), and the existence of clones may account for that. Two nuclear genotypes were found in seven out of nine plants sampled in Alcatrazes Island. The probability of identity was very low, suggesting that it is likely that individuals sharing the same genotype are ramets of the same genet. The significant negative G_{IS} value found at Alcatrazes Island also supports the presence of clonal propagation (Stoeckel and Masson 2014). Indeed, *Epidendrum fulgens* and many of its related species produce a large quantity of plantlets on their old inflorescences (Fig. 1c, Pinheiro and Cozzolino 2013). This strategy may confer a potential advantage in rock outcrop slopes, where storms and landslides constantly challenge the persistence of vegetation clumps. Clonal propagation

may also be advantageous in islands when conditions are not favorable for sexual reproduction, such as the absence of pollinators and mates and the absence of suitable places for seed germination (Barrett 1996; Lhuillier et al. 2006; Meloni et al. 2013).

Inbreeding depression was detected in all populations, indicated by the significant lower levels of seed viability found in self-pollinations (Fig. 3a). The occurrence of inbreeding depression may indicate outcrossing is the main reproductive strategy in both insular and continental populations, suggesting an ecological scenario where pollinators and mate availability are not affected by the insular environment. According to Pannell et al. (2015), inbreeding depression is not a fixed state in populations and may vary greatly between different colonization stages, such as long-established and recent colonized populations. Interestingly, inbreeding depression found at Alcatrazes Island is more variable than in the remaining populations, ranging from 0.0 to 60.0% of seed viability (Table 2, Fig. 3a), suggesting different responses to selfing. Several reproductive traits may favor the colonization and establishment in islands, such as perenniality, the capacity for self-fertilization or vegetative reproduction, or a capacity to be pollinated by generalist pollinators (Pannell et al. 2015). These reproductive traits are found in *E. fulgens* and may contribute to the resilience of the insular population facing demographic constraints caused by inbreeding depression. Moreover, the incidence of clonality and sexual reproduction may change across different habitat types within an island (Eckert et al. 2003; Meloni et al. 2013). Thus, further efforts of broader sampling along the Alcatrazes Island, covering different ecological conditions, will improve our knowledge on how clonal reproduction and inbreeding depression may provide reproductive assurance in harsh island environments.

Our data on interpopulation hand pollinations showed a significant decrease in seed fertility between Alcatrazes Island and remaining populations (Fig. 3b, Table 2). The decrease in seed fertility may indicate a postzygotic stage of reproductive isolation, caused primarily by genetic incompatibilities between mates (Scopece et al. 2010; Pinheiro et al. 2013). The evolution of postzygotic barriers is expected at final stages of speciation, when lineages have accumulated many genetic differences (Sasa et al. 1998; Bolnick and Near 2005; Turissini et al. 2018). Indeed, several studies have shown a positive relationship between the strength of each reproductive barrier and genetic distance (reviewed in Coyne and Orr 2004). However, more variable results are found in plants (reviewed by Baack et al. 2015), in which both pre- and postmating mechanisms may contribute to early stages of speciation. In this context, the high geographic isolation observed in islands may accelerate the genetic differentiation of island

organisms, increasing the effects of postzygotic isolation between insular and mainland populations.

Main genetic differences were observed at plastid markers (Fig. 2, Table 3), suggesting that cytonuclear incompatibilities may account for the decrease in fertility between different cytotypes, as observed in previous studies with different plant systems (Pinheiro et al. 2013; Barnard-Kubow et al. 2016). Indeed, these results suggest that rates at which reproductive isolation may evolve could be related to rates of organelle evolution among populations of the same species (Barnard-Kubow et al. 2016; Barnard-Kubow and Galloway 2017; Martin et al. 2017). Considering that genetic differentiation between continental and island populations was followed by higher levels of reproductive isolation, our results support the importance of outbreeding depression during the early stages of lineage diversification, when speciation process is still incomplete (Via 2009; Scopece et al. 2010). In further studies, investigating the role of neutral vs. adaptive processes in the accumulation of genetic incompatibilities by using high-throughput DNA sequencing (identification of selection signatures on particular loci), RNA (differential levels of gene expression) and environmental variables (Cruz et al. 2019; Osborne et al. 2019) will improve our understanding about the evolution of genetic incompatibilities in early stages of speciation and show the role of adaptive processes in island speciation, a topic of high interest in evolutionary research.

Information on Electronic Supplementary Material

Online Resource 1. Results of assignment test performed with the software Maverick.
Online Resource 2. Fruit set of hand pollination experiments.
Online Resource 3. Description of nine plastid microsatellite haplotypes of *Epidendrum fulgens*.

Author contributions FP and CPS participated in the study design and drafted the manuscript. GSV conducted the crossing experiments. CJNC analyzed the data from reproductive experiments. TCC genotyped the samples. All authors helped to draft the manuscript, read and approved the final version.

Data availability The datasets generated during and/or analyzed during the current study will be deposited on Dryad upon acceptance.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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