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Hybridization and introgression across different ploidy levels in the Neotropical orchids *Epidendrum fulgens* and *E. puniceoluteum* (Orchidaceae)

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Abstract

The hypothesis of gene flow between species with large differences in chromosome numbers has rarely been tested in the wild, mainly because species of different ploidy are commonly assumed to be reproductively isolated from each other because of instantaneous and strong postzygotic barriers. In this study, a broad-scale survey of molecular variation was carried out between two orchid species with different ploidy levels: *Epidendrum fulgens* ($2n = 2x = 24$ chromosomes) and *Epidendrum puniceoluteum* ($2n = 4x = 52$ chromosomes). To test the strength of their reproductive barriers, we investigated the distribution of genetic variation in sympatric and allopatric populations of these two species and conducted crossing experiments. Nuclear and plastid microsatellite loci were used to genotype 463 individuals from eight populations across the geographical range of both species along the Brazilian coastal plain. All six sympatric populations analysed presented hybrid zones, indicating that hybridization between *E. fulgens* and *E. puniceoluteum* is a common phenomenon. Bayesian assignment analysis detected the presence of F_1 and F_2 individuals and also signs of introgression, demonstrating a high potential for interspecific gene flow. Introgression occurs preferentially from *E. fulgens* to *E. puniceoluteum*. Pure parental individuals of both species display strong genotype–habitat associations, indicating that environment-dependent selection could be acting in all hybrid zones. This study suggests that hybridization and introgression are evolutionary processes playing a role in the diversification of *Epidendrum* and indicates the importance of investigations of hybrid zones in understanding reproductive barriers and speciation processes in Neotropical orchid species.

Keywords: *Epidendrum*, hybridization, introgression, orchid evolution, Orchidaceae, reproductive barriers

Introduction

Hybrid zones between cytotypes of different ploidy are of particular interest for studies of reproductive barriers in species complexes with mixed-ploidy (Thompson & Lumaret 1992; Petit *et al.* 1999; Chapman & Abbott 2010). The potential for gene exchange between taxa with large differences in chromosome numbers has long been recognized (Stebbins 1971), although it has rarely been tested in the wild (Petit *et al.* 1999; Chapman &

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Abbott 2010), mainly because species of different ploidies are commonly assumed to be reproductively isolated from each other because of strong postzygotic barriers (Coyne & Orr 2004). Recent theoretical and empirical studies have provided important evidence for the evolution of reproductive barriers in contact zones of diploid–polyploid species, including the possibility of introgression across species boundaries (Lord & Richards 1977; Ramsey & Schemske 1998; Petit *et al.* 1999; Aagaard *et al.* 2005; Shipunov *et al.* 2005; Pillon *et al.* 2007; Chapman & Abbott 2010).

Many hybrid zones in animals and plants are maintained by a balance of dispersal into the centre of the contact zones and intrinsic (endogenous) selection against hybrids (Barton & Hewitt 1985). In more complex hybridization scenarios involving exogenous selection and introgression across 'mosaic' hybrid zones (Rieseberg *et al.* 1999), the examination of genotype–habitat associations is of interest (Johnston *et al.* 2001). Divergent selection associated with differences in habitat can have a strong impact on patterns of reproductive isolation (RI) and gene flow between divergently adapted species in diploids and polyploids alike which will affect the impact and outcome of interspecific introgressive hybridization (Brennan *et al.* 2009). Tests for genotype–habitat associations are greatly favoured by the availability of species-informative molecular markers from different genomic compartments, most typically the nuclear and plastid DNA genomes in the case of plants (Soliva & Widmer 2003; Lexer *et al.* 2005; Moccia *et al.* 2007; Ståhlberg & Hedrén 2009).

Whether or not interspecific hybridization is important as a mechanism generating biological diversity in Orchidaceae is a matter of controversy. Several orchid hybrid zones have recently been documented and investigated with molecular markers, providing evidence either for (Aagaard *et al.* 2005; Bateman *et al.* 2008; Cortis *et al.* 2009; Bellusci *et al.* 2010) or against (Cozzolino *et al.* 2006; Moccia *et al.* 2007; Scopece *et al.* 2008) the potential role of hybridization in orchid evolution and diversification. In the case of Neotropical orchids, only limited and indirect evidence are available for the strength of RI between related species. Intermediacy in morphological characters, sexual compatibility between putative parental species and hybrid viability have all been used to infer the role of hybridization in orchid diversification in the Neotropics (Van der Pijl & Dodson 1966; Pansarin & Amaral 2008). Unfortunately, the genomic composition of putative hybrids remains unknown for most of these cases (but see Azevedo *et al.* 2006). Because information about genomic ancestry and admixture in Neotropical orchid hybrid zones is unavailable, the evolutionary processes underlying hybrid zone dynamics and genetic cohesion of parental species in species-rich Neotropical biomes remains unknown.

Epidendrum (subtribe Laeliinae) is the largest orchid genus in the Neotropics, comprising about 1500 species (Hágsater & Soto Arenas 2005). It is composed of several species complexes with many taxonomic uncertainties because of extensive morphological variability and wide variation in chromosome numbers. Hybridization has often been hypothesized as a driving force responsible for the origin of chromosomal and morphological variation in *Epidendrum* (Dunsterville 1979; Hágsater & Soto Arenas 2005; Pinheiro *et al.* 2009b). The existence of hybrid zones between *Epidendrum* species has been postulated by many authors based on morphological and distributional data (Van der Pijl & Dodson 1966; Dunsterville 1979; Hágsater & Soto Arenas 2005; Pansarin & Amaral 2008). The main evidence used to describe these hybrid swarms was the presence of individuals showing intermediate floral morphology relative to their putative parental species. Reproductive compatibility was reported for many co-occurring *Epidendrum* species, based on controlled interspecific crosses (Dunsterville 1979; Pansarin & Amaral 2008). In addition, a lack of pollinator specificity among *Epidendrum* species (Van der Pijl & Dodson 1966; Fuhro 2006; Pansarin & Amaral 2008) indicates a lack of pre-zygotic barriers suggesting that hybridization in natural populations could be a common phenomenon. To our knowledge, the numerous possible instances of hybridization among *Epidendrum* species have never been tested with rigorous molecular genetic methods.

Epidendrum fulgens and *Epidendrum puniceoluteum* are two enigmatic *Epidendrum* species that co-occur in many localities along the Brazilian sandy coastal plains in '*restinga*' vegetation (see Material and methods for details) throughout south (Rio Grande de Sul—24°50.886'S) and southeastern (Rio de Janeiro—28°12.538'S) Brazil (Fig. 1). The species are well differentiated for floral morphological traits and flower colour: orange sepals and petals and a yellow labellum in *E. fulgens*, red petals, sepals and labellum with a yellow labellum callus in *E. puniceoluteum* (Pinheiro & Barros 2006) (Fig. S1, Supporting Information). Phylogenetic analyses (Pinheiro *et al.* 2009b) indicated that *E. fulgens* and *E. puniceoluteum* are closely related species with divergent karyotype and chromosome numbers ($2n = 2x = 24$ and $2n = 4x = 52$, respectively). The existence of hybrids between these two species has been suspected ever since specimens with intermediate morphological characters were found in herbarium collections and natural populations where these species occur sympatrically (Fig. S1, Supporting Information) (Pinheiro & Barros 2006).

Fig. 1 Distribution map of *Epidendrum fulgens* and *Epidendrum puniceoluteum* based on field collection and herbarium material, and location of the populations studied. Allopatric populations of *E. fulgens* (*) and *E. puniceoluteum* (#) are indicated. PR, Paraná; RS, Rio Grande do Sul; SC, Santa Catarina; SP, São Paulo states.

In this study, an extensive investigation into genetic variation was carried out across the geographical range and overlapping zone of *E. fulgens* and *E. puniceoluteum*. In addition, the genome compatibility of parental species and the fertility of putative hybrid individuals were evaluated by manual crossing experiments. Specifically, we aimed to answer important questions related to the genetics and evolution of RI between these Neotropical orchids: (i) Do *E. fulgens* and *E. puniceoluteum* hybridize in the wild, as indicated by field and herbarium-based observations? (ii) If hybridization occurs, what is the genomic composition of hybrids when accessed using species-informative sets of nuclear and plastid DNA microsatellites? (iii) What do the genomic patterns tell us about the likelihood of hybridization and gene flow across ploidy levels? (iv) How strong are genotype–habitat associations in zones of sympatry, and what do they teach us about the role of ecology in currently maintaining the species barriers? (v) Which types of pre- and/or postzygotic barriers are expected to be the most important in RI between these taxa? The results allow us to discuss the evolutionary outcome of hybridization in diversification of this large and successful group of Neotropical orchids. The data also challenge the widely held view of 'instant isolation' among species of different ploidy (Coyne & Orr 2004).

Materials and methods

Plant species and habitat characteristics

Epidendrum fulgens and *Epidendrum puniceoluteum* are pollinated by butterflies, following a model of pollination by deceit, as there is no reward (nectar) for the pollinators (Moreira *et al.* 2008; Pinheiro unpublished results). Fuhro (2006) observed 29 butterfly species acting as potential pollinators of *E. fulgens*, indicating a generalist pollination system. Both species are self-compatible, but pollinators are necessary for pollen transfer. In addition, the two species share pollinators and have overlapping flowering phenologies (Fuhro 2006, Pinheiro unpublished results).

The species typically inhabit '*restinga*' vegetation, composed of a mosaic of different coastal plant communities along the Brazilian seashore (Araujo 1992). The structure and dynamics of this vegetation are affected by a sharp gradient of abiotic factors (e.g., salt spray, sand movement, soil moisture content, water availability) that decreases in intensity with increasing distance from the beach (Araujo 1992; Scarano 2002). Following Araujo (1992), '*restinga*' vegetation can be subdivided into several vegetation zones, based on their floristic composition. *Epidendrum fulgens* and *E. puniceoluteum* are mainly found in two different vegetation zones (following Araujo 1992): sand dunes that are not subject to flood, composed of shrubby vegetation, corresponding to *vegetation zone 2*, and sedge swamp communities flooded during most of the year, located in depressions between successive beach ridges, corresponding to *vegetation zone 5*. Vegetation zone 5 is located further inland than vegetation zone 2, but they are adjacent to each other (Fig. 2). Based on field observations, *E. fulgens* is more abundant in vegetation zone 2, whereas *E. puniceoluteum* is more abundant in vegetation zone 5.

Population sampling

Six putative hybrid zones were sampled, in which individuals of *E. fulgens*, *E. puniceoluteum* and putative hybrids were collected (Fig. 1). To test for habitat and genotype associations, the type of habitat in which individuals were collected, zone 2 or zone 5, was recorded. In addition, samples were collected from one allopatric population of *E. fulgens* and one allopatric population of *E. puniceoluteum*, to be analysed as reference populations (Fig. 1). In total, 463 individuals were sampled (Table 1). Individuals were collected randomly with a minimum sampling distance of 10 m. To extract a maximum amount of information regarding the detection of hybridization, we avoided collecting individuals resulting from vegetative reproduction (individuals growing

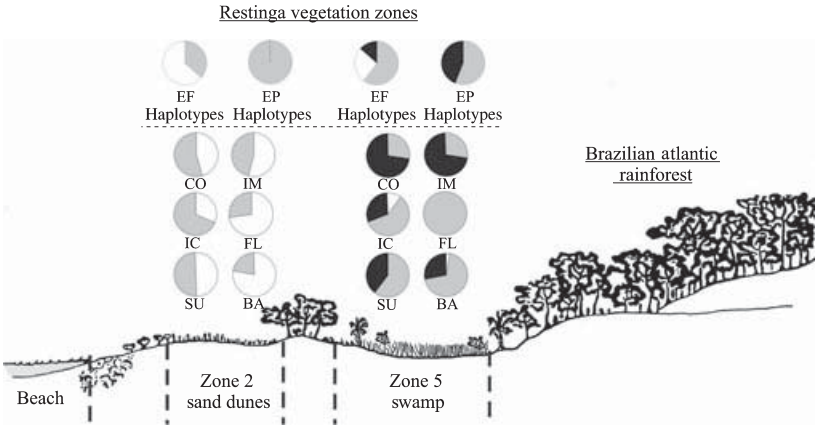

Fig. 2 Schematic representation of a transect from the beach to the slopes of the Brazilian Atlantic Rainforest (modified from Souza *et al.* 1997), indicating the location of vegetation zones 2 (sand dunes) and 5 (swamps) according to Araujo (1992), where the *Epidendrum* hybrid zones were sampled. A—Frequency of hybrids (grey) and pure individuals (*Epidendrum fulgens*—white; *Epidendrum puniceoluteum*—black) carrying the *E. fulgens* (EF) or *E. puniceoluteum* (EP) specific haplotypes, in each habitat. B—Frequency of pure parental species (*E. fulgens*—white; *E. puniceoluteum*—black) and hybrids (grey) in each population and habitat. See Table S1 (Supporting Information) for population codes and Table 4 for specific haplotypes of each parental species. Pure parental species and hybrids were classified considering Structure assignment results.

in clusters with many stems close to each other). Sample sizes, names and geographical co-ordinates of each population sampled are given in Table S1 (Supporting Information). For each individual plant collected, fresh leaves were dried in silica gel and stored at room temperature until laboratory procedures. Genomic DNA was extracted following procedures Pinheiro *et al.* (2008a).

Fertility estimates

Genome compatibility and fertility of *E. fulgens*, *E. puniceoluteum* and hybrid individuals (classified by Structure and NEWHYBRIDS results, see below) were estimated by testing seed viability of artificial crosses. Manual crossing experiments were performed at the orchid collection of the Instituto de Botânica (São Paulo, Brazil). All plants were transplanted from Ilha Comprida and Ilha do Cardoso populations and kept in collection for several years (before to be used in crossing experiments. Artificial crosses were conducted as described in Cafasso *et al.* (2005). Treatments were performed as follows: (i) intraspecific cross-pollination control; (ii) interspecific cross-pollination; (iii) between F1 hybrids; (iv) between backcrosses towards *E. puniceoluteum* (Bc-Ep); (v) between pure species and hybrids F1 (backcross pollination); (vi) between pure species and Bc-Ep; (vii) between F1 hybrids and Bc-ep. In total were used 80 flowers, from 20 plants (five of each pure species and hybrid classes-F1 and backcross). All crosses were conducted in both directions; each plant provided and received pollen. Seeds collected from mature capsules and stored at 4 °C.

Seed viability rates were evaluated by using tetrazolium test. This method uses 2,3,5-triphenyl tetrazolium chloride, in which viable embryos stained red (Lakon 1949). Seeds were placed in a 1% solution of triphenyl tetrazolium chloride and stored in darkness for 24 h at 30 °C. Samples of 300 seeds per fruit were analysed with an optical microscope. Percentage of viable seeds was calculated by dividing the number of viable embryos by the total number of embryos scored.

Nuclear and plastid microsatellite markers

In this study, we used nine nuclear microsatellite markers, six isolated from *E. fulgens* (markers EFF26, EFF29, EFF43, EFF45, EFF61, EFF70; Pinheiro *et al.* 2008a) and three isolated from *E. puniceoluteum* (markers Epp10, Epp18, Epp86; Pinheiro *et al.* 2008b). We choose the polymorphic markers with up to two alleles, based on cross-amplification tests performed by Pinheiro *et al.* (2009a). As cross-amplification tests contain important information about homology of the loci, our expectation was that *all* or *most* loci isolated from *E. fulgens* should amplify in *E. puniceoluteum*, as chromosome counts (Introduction) indicate that the latter was derived from an allo-polyploidization event involving the former as one parent. For the same reason, ca. *half* of the loci isolated from *E. puniceoluteum* should not be present in *E. fulgens*.

Five plastid microsatellite loci (Epcp01, Epcp02, Epcp04, Epcp08, Epcp09; Pinheiro *et al.* 2009c) were screened for identifying and characterizing plastid DNA haplotypes. Microsatellite loci were amplified by using polymerase chain reaction (PCR) in an Applied

Table 1 Characterization of populations of *Epidendrum fulgens*, *Epidendrum puniceoluteum* and hybrids, with nine nuclear and five plastid microsatellite markers, including the number of individuals sampled, number of alleles (A), allelic richness (AR), variance in allele size (Var), as well as expected (H_E) and observed (H_O) heterozygosities, the within population inbreeding coefficient f for nuclear microsatellites, the frequence of plastid DNA haplotypes found in each sample and the respective haplotype diversity (HD) for each population

	Nuclear microsatellites						Plastid microsatellites	
Species (sample size)	A	AR	Var	H_E	H_O	f	Haplotypes (frequency)	HD
Allopatric								
E. fulgens (25)	98	6.84	19.89	0.724	0.702	0.030	H2 (25)	0.000
E. puniceoluteum (27)	48	3.01	8.22	0.418	0.414	0.008	H11 (27)	0.000
Sympatric								
Ilha Comprida – SP								
E. fulgens (18)	75	5.97	14.67	0.642	0.616	0.041	H2 (18)	0.000
Hybrids (30)	77	7.44	11.82	0.621	0.605	0.025**	H2 (19), H9 (1), H11 (10)	0.388
E. puniceoluteum (21)	28	2.27	5.71	0.552	0.547	0.010	H2 (2), H9 (1), H11 (18)	0.161
Ilha do Cardoso—SP								
E. fulgens (20)	70	5.45	15.69	0.604	0.586	0.036	H2 (20)-	0.000
Hybrids (53)	81	7.99	13.68	0.631	0.614	–0.026**	H2 (29), H11 (24)	0.404
E. puniceoluteum (9)	16	1.71	1.19	0.373	0.136	0.639*	H11 (9)	0.000
Ilha de Superagui – PR								
E. fulgens (17)	77	6.38	20.89	0.649	0.586	0.084	–	–
Hybrids (27)	72	7.14	11.19	0.585	0.617	–0.054	–	–
Species (sample size)	A	AR	Var	H_E	H_O	f	Haplotypes (frequency)	HD
E. puniceoluteum (6)	18	2.00	3.88	0.512	0.500	0.027	–	–
Ilha do Mel—PR								
E. fulgens (23)	71	5.55	15.16	0.617	0.652	0.084	–	–
Hybrids (27)	82	7.95	14.08	0.620	0.664	–0.056	–	–
E. puniceoluteum (18)	31	2.48	9.10	0.446	0.412	0.076	–	–
Florianópolis—SC								
E. fulgens (35)	110	7.01	18.57	0.740	0.736	0.003	H2 (31), H3 (2), H6 (2)	0.044
Hybrids (20)	87	8.97	14.56	0.665	0.594	0.107*	H2 (3), H3 (1), H6 (1), H11 (15)	0.245
Imbituba—SC								
E. fulgens (38)	105	6.85	16.54	0.759	0.689	0.093**	H1 (3), H2 (34), H5 (1)	0.040
Hybrids (38)	93	8.58	8.29	0.684	0.663	0.028	H1 (5), H2 (12), H5 (1), H11 (20)	0.455
E. puniceoluteum (11)	28	2.62	3.03	0.472	0.375	0.222**	H11 (11)	0.000
Overall = 463 individuals								

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

Biosystems 2700 thermocycler (Applied Biosystems, Foster City, CA, USA) following the protocol described by Pinheiro *et al.* (2008a). The conditions were maintained constant for all loci to maximize standardization. Genotyping procedures were performed on a 3130 DNA Sequence Analyser (Applied Biosystems) using LIZ (500) standard size (Applied Biosystems) and GeneMapper v3.7 software (Applied Biosystems).

Data analysis

Nuclear admixture analysis and assignment tests. . The classification of individuals based on morphological characters was difficult, especially in sympatric populations, because of the extensive morphological variability found in both species and putative hybrids (Fig. S1). Thus, individuals were classified as *E. fulgens*, *E. puniceoluteum* and hybrids using nuclear molecular markers, analysed by Bayesian assignment tests. To achieve this, allopatric populations of each species were used as reference samples of pure individuals of *E. fulgens* and *E. puniceoluteum*. To estimate nuclear admixture proportions and patterns of introgression, two Bayesian clustering methods were performed, in the software: Structure version 2.2 (Pritchard *et al.* 2000) and NEWHYBRIDS version 1.1 beta (Anderson & Thompson 2002). Analyses were performed separately for each hybrid zone, in each case including the specimens from the allopatric populations as reference samples for each species. In the model implemented in Structure, the posterior probability (q) is the proportion of a given genotype originating from each of cluster categories (K).

In this study $K = 2$ model was used, because we assume the two species contributes to the gene pool of the sample. The model implemented in NEWHYBRIDS assumes that samples analysed are composed by two pure parental species and hybrids. Under this model, q describes posterior probabilities for each individual, which are classified as parental purebreds, F1, F2 and backcross categories. With Structure, calculations were performed under admixture model. A burn-in of 50 000 steps followed by run lengths of 300 000 were used in each program. The optimal threshold value (Tq) for the q was choose based on simulations performed as described in Vähä & Primmer (2006) and Burgarella *et al.* (2009). Based on the results from simulations (Tables S2 and S3, Supporting Information), Structure was used to classify individuals among the two parental species and hybrids, using a threshold of $q \geq 0.90$ to classify pure individuals of *E. fulgens*, $q \leq 0.10$ to classify pure individuals of *E. puniceoluteum* and $0.10 \leq q \leq 0.90$ to classify hybrids. NEWHYBRIDS was used to classify hybrids, which were previously classified by Structure, in classes (F1, F2 and backcrosses), using a threshold value of $q = 0.75$; individuals with $q < 0.75$ remained unassigned (details of the simulations can be obtained from the authors request).

Nuclear and plastid genetic diversity. The nuclear microsatellite loci were characterized in the *E. fulgens*, *E. puniceoluteum* and their hybrids based on the number of alleles, variance in allele size, expected and observed heterozygosity standardized genetic differentiation (G'_{ST}—Hedrick 2005) and the inbreeding coefficient (f—Weir & Cockerham 1984), calculated for each locus using the programs FSTAT (Goudet 1995) and MSA (Dieringer & Schlötterer 2003). The software GENEPOP on the web (Raymond & Rousset 1995) was used to test departures from Hardy–Weinberg equilibrium (HWE) for each locus within each species and hybrids. Genotyping errors attributed to stutter, short allele dominance and null alleles were checked using software MICRO-CHECKER (Van Oosterhout *et al.* 2004). Populations were characterized by number of alleles, allelic richness (a more sensitive diversity parameter than H_E or H_O; Lexer *et al.* 2005), variance in allele size, expected and observed heterozygosity and the within population inbreeding coefficient (f) calculated by FSTAT. Departures from HWE for each population were tested using exact tests in software GENEPOP on the web. To explore the existence of clones in both species and hybrids, we performed an analysis using Gimlet software (Valiére 2002).

Diversity of plastid DNA markers was accessed for populations Ilha Comprida, Ilha do Cardoso, Pontal do Sul, Florianópolis, Imbituba and Torres, using number of haplotypes observed in each sample, haplotype diversity and θ (theta) among species and within each species estimated by ARLEQUIN 3.01 (Excoffier *et al.* 2005). A haplotype network was built based on plastid DNA haplotypes in the software Network v. 4.5.1.0 (http://www.fluxus-engineering.com) applying median-joining option (Bandelt *et al.* 1999). It uses the maximum parsimony criteria to reconstruct all possible shortest least complex phylogenetic trees. Singleton haplotypes were excluded to simplify the network.

To explore patterns and consequences of cytonuclear incompatibilities in interspecific hybridization in *Epidendrum* species, departures from random cytonuclear associations (Arnold 1993), nuclear admixture proportions, based on Structure results, and plastid DNA haplotype data were compared using the nonparametric Spearman rank correlations with the software SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Habitat associations among purebred individuals and hybrids

Individuals classified as pure *Epidendrum fulgens* and pure *Epidendrum puniceoluteum* based on nuclear markers differed strongly in their habitat preferences (Fig. 2). All individuals classified as pure *E. puniceoluteum* were collected in the swamp habitat (zone 5), and almost all pure *E. fulgens* specimens were only found growing in sand dunes (zone 2), whereas hybrids were found in both habitats (Fig. 2). In the Florianópolis population, where the swamps are restricted to a few small patches, pure *E. puniceoluteum* individuals were not identified. In the sand dune habitat, *E. fulgens* plastid DNA haplotypes were found in individuals classified as *E. fulgens* and as hybrids based on the nuclear genetic data, whereas haplotypes specific to *E. puniceoluteum* were found exclusively in hybrids (Fig. 2). In the swamp habitat, haplotypes specific to *E. fulgens* were found in both species and hybrids, whereas haplotypes specific to *E. puniceoluteum* were found in pure individuals of *E. puniceoluteum* and hybrids only (Fig. 2).

Genome compatibility and hybrid fertility

Artificial crosses showed that the two parental species produced fruits with a highly viable seeds in intraspecific and interspecific crosses (Table 2). F1 hybrids and backcrosses towards *E. puniceoluteum* only produced fertile seeds when they were pollinated by pure *E. puniceoluteum* plants. None of the crosses between *E. fulgens* and hybrids produced fruits. Fruits were not formed when F1 hybrids and backcross individuals acted as pollen donors (Table 2).

Donor	Recipient			
	E. fulgens	F1 Hybrid	Bc-Ep	*E. puniceoluteum*
E. fulgens	95.4 (±6.3)	0 (±0)	0 (±0)	88.7 (±12.1)
F1 Hybrid	0 (±0)	0 (±0)	0 (±0)	0 (±0)
Bc-Ep	0 (±0)	0 (±0)	0 (±0)	0 (±0)
E. puniceoluteum	82.2 (±3.8)	62.4 (±2.2)	73.6 (±9.2)	98.7 (±14.1)

Table 2 Percentage of viable seeds produced from hand pollination of *Epidendrum fulgens*, *Epidendrum puniceoluteum*, F1 hybrids and backcrosses towards *E. puniceoluteum* (Bc-Ep). Standard errors in parentheses

Genetic composition of hybrid zones

Using simulated data, Structure was first employed to classify individuals using a threshold of $q = 0.90$, then NEWHYBRIDS was used to assign plants to different hybrid classes using a threshold value of $q = 0.75$ (Tables S2 and S3). Power and accuracy (Vähä & Primmer 2006) showed values higher than 0.90 for identification of purebreds and hybrids, using both Structure and NEWHYBRIDS, indicating a satisfactory overall performance as well.

Bayesian assignment results indicated that the allopatric populations of *E. fulgens* and *E. puniceoluteum*, used as reference populations, were composed exclusively of purebreds (Structure threshold q-value ≥ 0.900 or $q \leq 0.100$, respectively) (Figs 3 and S2). For most individuals of *E. fulgens* classified in the field as pure, species status was confirmed with Structure with a threshold of $q \geq 0.90$ (Fig. 3). In contrast, many individuals classified in the field as *E. puniceoluteum* showed intermediate q-values ($0.10 \leq q \leq 0.90$) with Structure, indicating hybrid ancestry. The existence of hybrids was confirmed in all six sympatric populations (Figs 3 and S2). Two hundred and five individuals were classified as hybrids (Table 1), ranging from 27 (39.71%) in Ilha do Mel to 53 (64.63%) in Ilha do Cardoso.

One hundred and four individuals out of 205 were unequivocally assigned to different hybrid classes (F1, F2 and backcrosses) with NEWHYBRIDS using q-values ≥ 0.75 (Figs 3 and S2). In total, 32 F1, 67 backcrosses with *E. puniceoluteum* and five F2 genotypes were identified, (Figs 3 and S2). The hybridization patterns recovered indicate unidirectional introgression across the species barrier, and variation in hybrid genomic composition among populations. Whereas F1 genotypes were frequent in the Ilha do Cardoso population, backcrosses with *E. puniceoluteum* were more abundant in the remaining hybrid zones (Figs 3 and S2). No backcrosses towards *E. fulgens* were detected in the studied populations. There is no apparent pattern of specific morphological features in the depicted hybrid classes, because F1 and backcross individuals both showed intermediate characteristics between the parental species and more similar morphological traits with *E. puniceoluteum* (Figs 3 and S2).

Genetic variation at nuclear microsatellite loci

High levels of polymorphism were observed in most of the genotyped loci, with up to 30 alleles per locus, expected heterozygosities (H_E) ranged from 0.005 to 0.908 and observed heterozygosities (H_O) from 0.005 to 0.917 (Table 3). The variance in allele size ranged from zero to 85.2. The allelic richness revealed increased variability in hybrids. Three loci displayed significant deviation from HWE in *E. fulgens* and two in *E. puniceoluteum*, possibly reflecting occasional departures from random mating, because genotyping errors, such as null alleles, were ruled out by MICRO-CHECKER tests. Significant departures from HWE occurring across both species and hybrids were not observed for any loci (Table 3). Significant genetic differentiation was found between the two species in the entire data set and in each locality (whole data set: $G'_{ST} = 0.75$, P-value = 0.001; minimum $G'_{ST} = 0.40$, Florianópolis; maximum $G'_{ST} = 0.91$, Ilha do Cardoso). Intraspecific differentiation was lower in *E. fulgens* than in *E. puniceoluteum* ($G'_{ST} = 0.235$, P-value = 0.001, and $G'_{ST} = 0.290$, P-value = 0.001, respectively), and inbreeding coefficients (Table 3) were low in both species ($f = 0.05$ and $f = 0.06$, respectively).

Genetic diversity in Epidendrum *populations*

Genetic diversity was higher in *E. fulgens* than in *E. puniceoluteum* across all populations and parameters (Table 1), which probably reflects differences in population sizes found between the species (higher in *E. fulgens*). *Epidendrum fulgens* and *E. puniceoluteum* from Imbituba displayed significant departures from HWE because of heterozygote deficits. Three hybrid zones deviated from HWE, indicating departures from random mating. No instances of clonality (=duplicated genotypes) were found, across the sampling distance, in any of the populations.

Plastid DNA diversity and haplotype network

Eleven haplotypes were identified based on the five plastid microsatellites analysed. Four haplotypes were removed from the subsequent analysis because they

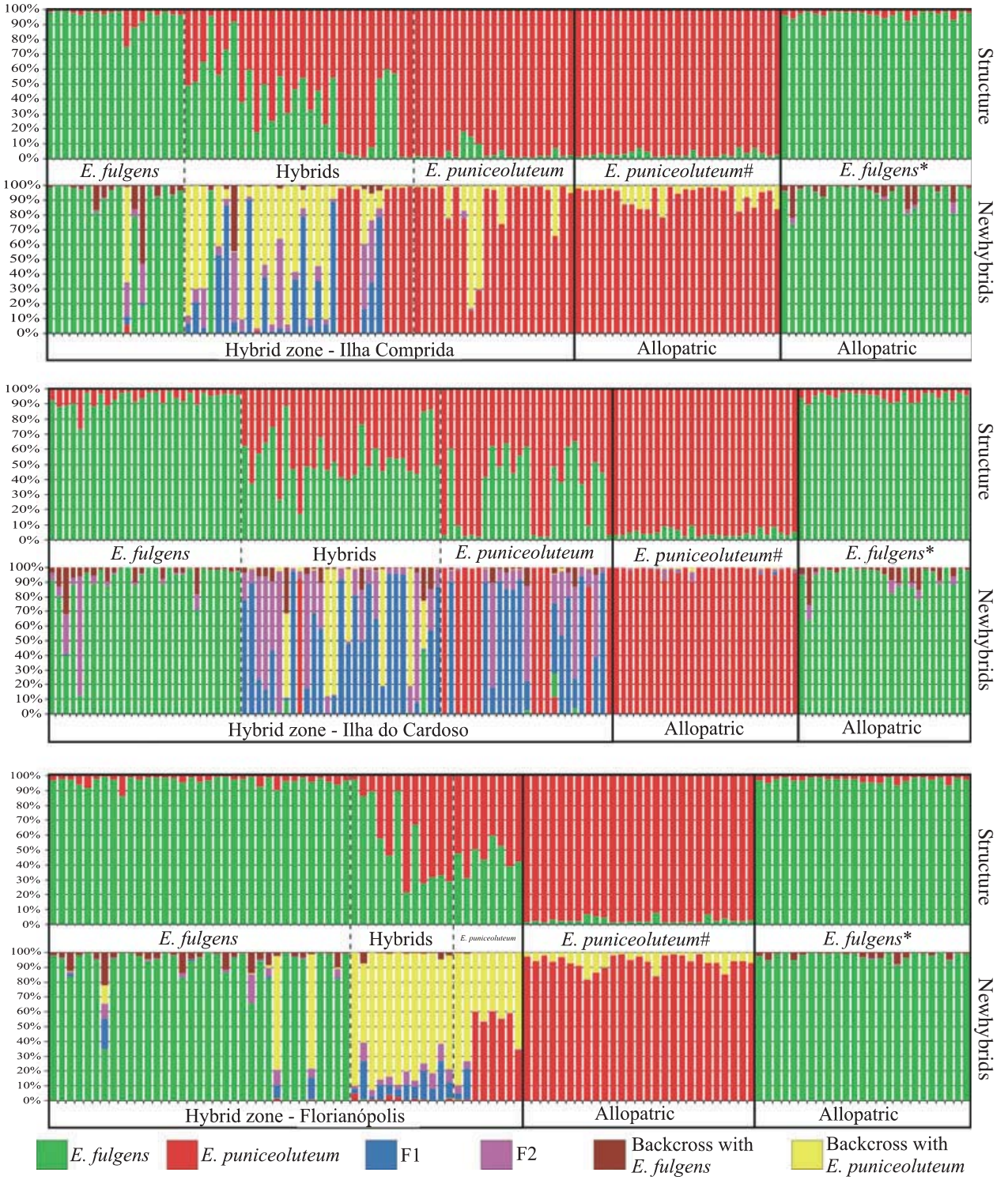

Fig. 3 Posterior probabilities (*q*) for Ilha Comprida, Ilha do Cardoso and Florianópolis natural hybrid zones analysed with Structure and NEWHYBRIDS. Hybrid zone and allopatric localities of *Epidendrum fulgens* in Torres (*) and *Epidendrum puniceoluteum* in Pontal do Sul (#) are delimited by solid lines. Individuals identified in the field, based on morphological characters, are delimited by dashed lines. Each vertical bar represents an individual. The proportion of colour in each bar represents an individual's assignment probability, according to different categories (pure parental species, hybrid F1, F2 and backcrosses). See Fig. 1 for details of geographical position of each locality.

Table 3 Genetic variability at nine nuclear microsatellite loci in *Epidendrum fulgens*, *Epidendrum puniceoluteum* and hybrids, including locus name, number of alleles (A), variance in allele size (Var), expected (H_E) and observed (H_O) heterozygosity, standardized genetic differentiation measure (G'_{ST}) and inbreeding coefficient (f) for each locus

	E. fulgens						Hybrids						*E. puniceoluteum*					
Locus	A	Var	H_E	H_O	G'_{ST}	f	A	Var	H_E	H_O	G'_{ST}	f	A	Var	H_E	H_O	G'_{ST}	f
Epp10	22	18.6	0.903	0.806*	0.395	0.096	20	18.2	0.810	0.701**	0.269	0.136	9	8.4	0.508	0.378	0.299	0.220
Epp18	30	85.2	0.901	0.845**	0.539	0.073	24	24.2	0.804	0.748	0.204	0.068	12	1.7	0.167	0.183	0.044	−0.152
EFF26	7	1.0	0.524	0.506	0.499	0.043	9	2.1	0.725	0.823**	0.363	−0.132	6	0.5	0.123	0.043	0.372	0.224
EFF29	23	16.6	0.907	0.891	0.335	0.025	21	23.6	0.897	0.917	0.341	−0.020	17	15.5	0.717	0.708*	0.489	0.027
EFF43	7	1.4	0.349	0.351	0.310	0.018	7	0.2	0.137	0.132	0.047	0.024	2	0.3	0.095	0.091	0.561	0.019
EFF45	6	0.5	0.539	0.519	0.138	0.041	4	0.3	0.258	0.112**	0.038	0.562	2	0.2	0.103	0.099	0.556	0.020
EFF61	5	0.3	0.172	0.156	0.142	0.120	6	0.3	0.420	0.463	0.225	−0.103	2	0.2	0.401	0.383	0.419	−0.090
EFF70	22	17.6	0.908	0.891	0.281	0.006	21	15.2	0.834	0.863	0.167	−0.033	13	7.1	0.488	0.370**	0.372	0.093
Epp86	16	12.2	0.885	0.797**	0.286	0.072	16	6.4	0.829	0.855	0.145	−0.031	7	2.6	0.568	0.532	0.317	0.096
Overall	15.33	17.04	0.676	0.640	0.235	0.052	14.22	10.06	0.635	0.624	0.144	0.017	7.78	4.00	0.331	0.290	0.261	0.065

*$P < 0.05$; **$P < 0.005$, exact tests for departure from Hardy–Weinberg equilibrium.

were only found in single individuals (singletons). Genetic diversity in populations (Table 1) ranged from 0.0 (one haplotype) to 0.455 (four haplotypes). Great differentiation among species ($\theta = 0.89$) and low genetic structure within species (*E. fulgens*: $\theta = 0.03$ and *E. puniceoluteum*: $\theta = 0.05$) were detected. Two major groups could be recognized in the haplotype network (Fig. 4), one of which contained five haplotypes typical of *E. fulgens* (individuals with $q \geq 0.900$), whereas the other one was composed of two haplotypes from *E. puniceoluteum* (individuals with $q \leq 0.100$). Hybridization occurred in both directions (with both species acting as male and female parents), because all types of haplotypes could be observed in hybrid individuals (Table 4). Haplotypes H2, typical of *E. fulgens*, and H11 typical of *E. puniceoluteum*, were present in F1, F2 and backcross individuals. There was no evidence of plastid DNA introgression into *E. fulgens*, whereas two individuals of *E. puniceoluteum* carried haplotypes typical of *E. fulgens* (H2), thus indicating introgression of the plastid DNA. Extensive variation in nuclear admixture proportions was detected in hybrids carrying *E. fulgens* ($q = 0.051$–0.988) and *E. puniceoluteum* haplotypes ($q = 0.014$–0.884), indicating variation in hybrid ancestry (Table 4), and a significant correlation was detected between nuclear and plastid genomic composition (Spearman's $r = -0.695$, $P = 0.000$), suggestive of cytonuclear incompatibilities.

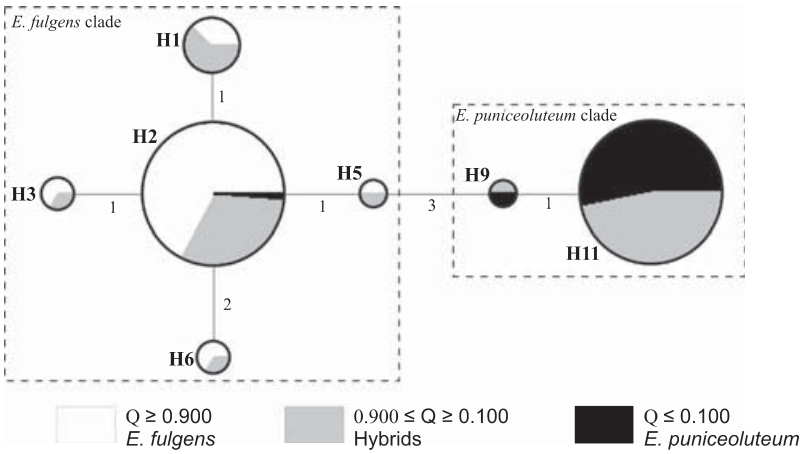

Fig. 4 Median-joining network among plastid DNA haplotypes. The haplotypes are indicated by filled circles, the size of each circle being proportional to the observed frequency of each type. The colours indicate the amount of individuals classified according Structure assignment probabilities (q). The number of mutations required to explain transitions among haplotypes is indicated along the lines of the network.

Table 4 Plastid DNA haplotypes (H) found in 345 individuals from four hybrid zones, including indication about haplotype sharing among taxa, frequencies in both species and hybrids considering Structure and NEWHYBRIDS assignment probabilities and range of nuclear admixture proportions for plants carrying each haplotype (only with Structure admixture proportions)

		Frequencies (Structure assignment results)				Frequencies (NEWHYBRIDS assignment results)		
H	Haplotype group	*Epidendrum fulgens* (N = 136)	Hybrids (N = 141)	*Epidendrum puniceoluteum* (N = 68)	Structure admixture range	F1	F2	BP
H1	*E. fulgens*	3	5	0	0.539–0.979			
H2	*E. fulgens*	128	63	2	0.051–0.991	18	4	30
H3	*E. fulgens*	2	1	0	0.624–0.990			
H5	*E. fulgens*	1	1	0	0.516–0.940			
H6	*E. fulgens*	2	1	0	0.598–0.988			
H9	*E. puniceoluteum*	0	1	1	0.014–0.265			
H11	*E. puniceoluteum*	0	69	65	0.012–0.884	14	1	37

For Structure assignment probabilities, were considered pure *E. fulgens*, pure *E. puniceoluteum* and hybrids individuals with posterior probabilities of $q \geq 0.90$, $0.10 \leq q \leq 0.90$ and $q \leq 0.10$, respectively.
In NEWHYBRIDS assignment results, only hybrid classes with individuals showing posterior probabilities of $q \geq 0.75$ were included.

Discussion

Hybridization in Epidendrum *sympatric populations*

Hybridization was detected in all sympatric populations analysed (Figs 3, S2), confirming the previous hypotheses of hybridization between *Epidendrum fulgens* and *Epidendrum puniceoluteum* based on field and herbarium observations of floral intermediate morphology (Pinheiro & Barros 2006). Experimental crosses performed in a common garden environment confirm the genome compatibility between *E. fulgens* and *E. puniceoluteum* and that unidirectional introgression towards the polyploid species is possible. Nuclear and plastid microsatellite loci revealed the admixture proportions and genetic architecture of all hybrid zones (Figs 3 and S2) and were able to depict specific plastid DNA variants of *E. fulgens* and *E. puniceoluteum* (Fig. 4). The nuclear data indicate F1 and backcrosses towards *E. puniceoluteum* as the most frequent genotypic classes in the hybrid zones. Hybridization occurs in both directions, and F1 hybrids can act as pollen receptors to produce backcrossed individuals with *E. puniceoluteum* (Table 4). Incongruent data were obtained only for the presence of F2 hybrids, as no fertile seeds were observed in crosses between F1 plants, but five F2 plants were identified using nuclear markers, based on NEWHYBRIDS results. More crosses and a higher number of loci will be needed to clarify the existence of F2 hybrids in natural populations.

Pure individuals from both species showed high levels of genetic differentiation (G'_{ST}) and several fixed plastid haplotypes (Fig. 4; Table 4), suggesting that the hybrid zones may have arisen by secondary contact, after enough time had passed for the differentiation observed to accumulate. Different habitat associations were detected between parental genotypes (Fig. 2), suggesting that niche divergence contributes to species cohesion as a potential barrier limiting gene flow (see Johnston *et al.* 2001). The introgression between *E. fulgens* and *E. puniceoluteum* challenges the widely held view of 'instant isolation' among species of different ploidy (Coyne & Orr 2004), consistent with observations for other orchid species (see Discussion below) and plant groups (reviewed by Ramsey & Schemske 1998; Chapman & Abbott 2010).

The data set not only captures the architecture of the hybrid zones, but also allows conclusions to be drawn regarding the ecological processes shaping current patterns of gene flow between *E. fulgens* and *E. puniceoluteum*, the strength of pre- and postzygotic barriers between these species and the role of hybridization in *Epidendrum* diversification.

Hybridization between individuals of different ploidy

The recombinant hybrid classes found between *E. fulgens* and *E. puniceoluteum* (backcrosses) indicate backcrossing only towards the tetraploid species: *E. puniceolutem*. Our results are similar to those obtained by two studies on orchid hybridization in the genus *Dactylorhiza*: *D. incarnata* subsp. *cruenta* ($2n = 2x = 40$) and *Dactylorhiza lapponica* ($2n = 4x = 80$) (Aagaard *et al.* 2005) and *Dactylorhiza maculata* subsp. *fuchsii* and *D. maculata* subsp. *maculata* (Ståhlberg & Hedrén 2009). In these studies, hybrid zones are composed of first generation hybrids and backcrosses towards the polyploid parental species. Cross-amplification tests using nuclear microsatellite loci developed specifically for *E. fulgens*

and *E. puniceoluteum* show that all loci developed for *E. fulgens* (diploid) can be successfully amplified in *E. puniceoluteum* (tetraploid), but only 60% of the markers developed for *E. puniceoluteum* could be amplified in *E. fulgens* (Pinheiro *et al.* 2009a). All this suggests that *E. puniceoluteum* is an allotetraploid having *E. fulgens* as one parent. The presence of genomic elements of *E. fulgens* in the genome of *E. puniceoluteum* could facilitate chromosome pairing between F1 hybrids and *E. puniceoluteum*, but not in the opposite direction. The same pattern was found in hybrid zones between diploid *D. incarnata* and allotetraploid *D. lapponica*, where introgression was found towards the allotetraploid species *D. lapponica* (Aagaard *et al.* 2005). The role of a possible existence of a triploid bridge between *E. fulgens* and *E. puniceoluteum* in mediating gene exchange and introgression needs further clarification, because there are examples in the literature showing introgression occurring towards the diploid (Ramsey & Schemske 1998) and towards the polyploid species (Stebbins 1971; Chapman & Abbott 2010). Cytogenetic studies into the meiotic behaviour and GISH analyses (genomic *in situ* hybridization) of both parental species and hybrids would help to clarify chromosome homologies and parentage.

Incomplete pre- and postzygotic isolation

Epidendrum fulgens and *E. puniceoluteum* are food-deceptive orchids pollinated by many species of butterflies (Fuhro 2006; Moreira *et al.* 2008; Pansarin & Amaral 2008; Pinheiro unpublished results). Pollinator sharing is commonly observed in food-deceptive orchids (Cozzolino *et al.* 2004). Low values of inbreeding coefficients and low to moderate values of genetic differentiation (Table 3) were observed, indicating weak genetic structure among populations within species. These patterns are in accordance with those expected for species without reward, because this process limits geitonogamous pollination, promoting outcrossing and reducing population differentiation (Soliva & Widmer 2003; Cozzolino & Widmer 2005). Thus, outcrossing plays a major role in parental species reproduction (low f values), hybrid formation and introgression, indicating that premating isolation in *Epidendrum* hybrid zones is relatively weak.

Hybridization and breakdown of species boundaries have been observed for many co-occurring deceptive orchid species (species with weak or absent pollinator specificity) in the Mediterranean region (Cozzolino *et al.* 2004; Moccia *et al.* 2007; Scopece *et al.* 2008; and references therein). In Mediterranean deceptive orchids, gene flow among co-occurring species is limited by late-acting postzygotic reproductive barriers, such as chromosomal rearrangements (Cozzolino *et al.* 2004) promoting F1 hybrid sterility and preventing backcrossing. However, in *Epidendrum* hybrid zones we observed weaker postzygotic barriers to gene flow when compared with Mediterranean food-deceptive orchids (Cozzolino *et al.* 2006; Moccia *et al.* 2007; Scopece *et al.* 2007, 2008), because a high number of viable seeds were produced by hybrid individuals when receiving pollen from *E. puniceoluteum*. This is in agreement with the large proportion of backcrosses to *E. puniceoluteum* observed in natural hybrid zones.

Our results indicate strong male sterility in F1 hybrids and backcrosses, because fruits or viable seeds were absent in crosses when hybrids act as pollen donors, suggesting low quality of the hybrid pollen. In orchids, hybrid pollen sterility is commonly observed (Peakall *et al.* 1997; Scopece *et al.* 2008), mainly because of irregular microsporogenesis (Stort 1984). The incomplete postmating reproductive isolation between *E. fulgens* and *E. puniceoluteum* suggests that extrinsic (environmentally dependent) selective factors may act in limiting gene flow and in keeping species boundaries between these species.

Habitat and genotype associations

Pure parental individuals of both species display strong association with different habitats in 'restinga' vegetation (zone 2: sand dunes and zone 5: swamps), suggesting a possible role of environment-dependent selection in all hybrid zones. Similar patterns were observed in *Ranunculus* and *Iris* (He *et al.* 1999; Johnston *et al.* 2001) suggesting that species with high tolerance to flooding are able to co-occur with another dominant species that is intolerant to flooding (Johnston *et al.* 2001). Previous studies have shown that environmental-dependent selection can be important in the maintenance of parental species barriers in hybrid zones (Cruzan & Arnold 1993; He *et al.* 1999; Johnston *et al.* 2001; Ståhlberg & Hedrén 2009). Tolerance to salinity stress and flooding are characteristics playing an important role in hybrid zones in coastal habitats (Johnston *et al.* 2001; Van Zandt *et al.* 2003). Further investigation is needed to shed light on the role of abiotic factors in species cohesion in *Epidendrum* hybrid zones. Reciprocal transplant experiments between pure individuals of *E. fulgens* and *E. puniceoluteum* will provide a measure of divergent natural selection occurring in sand dunes and swamps (Schluter 2000).

Introgression and increase of ecological amplitude

Transfer of adaptation among species with different cytotypes may be promoted by interspecific gene flow (Kim *et al.* 2008; Chapman & Abbott 2010). In

Helianthus (Asteraceae), hybrid zones Rieseberg *et al.* (1996) demonstrated that genomic composition of hybrids is constrained by interactions between co-adapted genes from parental species; however, the adaptive evolution of hybrids may be improved by some gene combinations. The introgression of fitness-related genes across ploidy barriers between the diploid *Senecio squalidus* and the tetraploid *Senecio vulgaris* (Asteraceae) produced a variant form of *S. vulgaris* with flowers more attractive to pollinators and a higher outcrossing rate (Kim *et al.* 2008; Chapman & Abbott 2010).

Epidendrum hybrid genotypes inhabited sand dunes and swamps, with extensive overlap with their parental species in all sympatric zones (Fig. 2). High genetic diversity was observed in hybrids, indicated by allelic richness, probably originating from combination of alleles from the two parental species. Large nuclear admixture range proportions, indicated by the Structure and NEWHYBRIDS results, were also found in hybrids, suggesting a connection between extensive genetic admixture and the ability to grow in different habitats, extending the ecological amplitude found in hybrids relative to the parental species (Lexer *et al.* 2005). The '*restinga*' vegetation is composed chiefly of plant species with broad ecological amplitude. In these communities many adverse environmental conditions are common, such as high levels of salinity, flooding, drought and lack of soil nutrients (Scarano 2002). Positive interactions among plants (nurse and pioneer species) are important in the structure and function of coastal swamps and sandy vegetation (Scarano 2002). Hybridization generating introgression towards *E. puniceoluteum* can be another positive interaction occurring in this ecosystem. In this context, introgression of genomic elements from *E. fulgens* into *E. puniceoluteum* would allow introgressed individuals to grow in both swamps and sand dunes. Transplant experiments could be used to test this hypothesis of increased ecological amplitude in introgressed individuals, using fitness measures taken from individuals with the same genetic composition (reproduced vegetatively) growing in sand dunes and swamps (Schluter 2000).

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This study is part of Fábio Pinheiro's PhD research on hybridization, speciation and the maintenance of species boundaries in Neotropical orchids, carried out at the University of São Paulo, Brazil. Dr Fábio de Barros works with taxonomy and morphological variation of Neotropical orchids, and is advisor of Fábio Pinheiro's dissertation. Dr Diogo Meyer is broadly interested in the ecological and evolutionary genetics of mammalian vertebrates. Dr Clarisse Palma da Silva is interested on evolution and diversification of Neotropical plants. Dr Michael F. Fay studies population genetic and cytogenetic aspects of plant evolution with a focus on conservation. Dr Rogério M. Suzuki work in orchid micropropagation and *ex situ* conservation of endangered species. Dr Christian Lexer's main interest is on the genetics of speciation in selected plant groups. The main interests of Dr Salvatore Cozzolino are ecology, evolution and conservation of orchids.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 *Epidendrum* populations sampled for the present study, including population names (codes), sample size (*n*) and geographical co-ordinates

Table S2 Results of Structure assignment analyses with 10 simulated samples of 60 individuals each ($N = 600$), without hybrids (simulated HP = 0%), and 10 simulated samples of 100 individuals each ($N = 1000$), including hybrids (simulated HP = 40%)

Table S3 Results of NEWHYBRIDS analyses with 10 simulated samples of 100 individuals each ($N = 1000$), considering only hybrid classes F1, F2, backcross with *Epidendrum fulgens* (BF) and backcross with *Epidendrum puniceoluteum* (BP)

Fig. S1 Morphological variation of flowers in individuals of *Epidendrum fulgens*, *Epidendrum puniceoluteum* and hybrids.

Fig. S2 Posterior probabilities (*q*) for Ilha de Superagui, Ilha do Mel and Imbituba natural hybrid zones analysed with Structure and NEWHYBRIDS.