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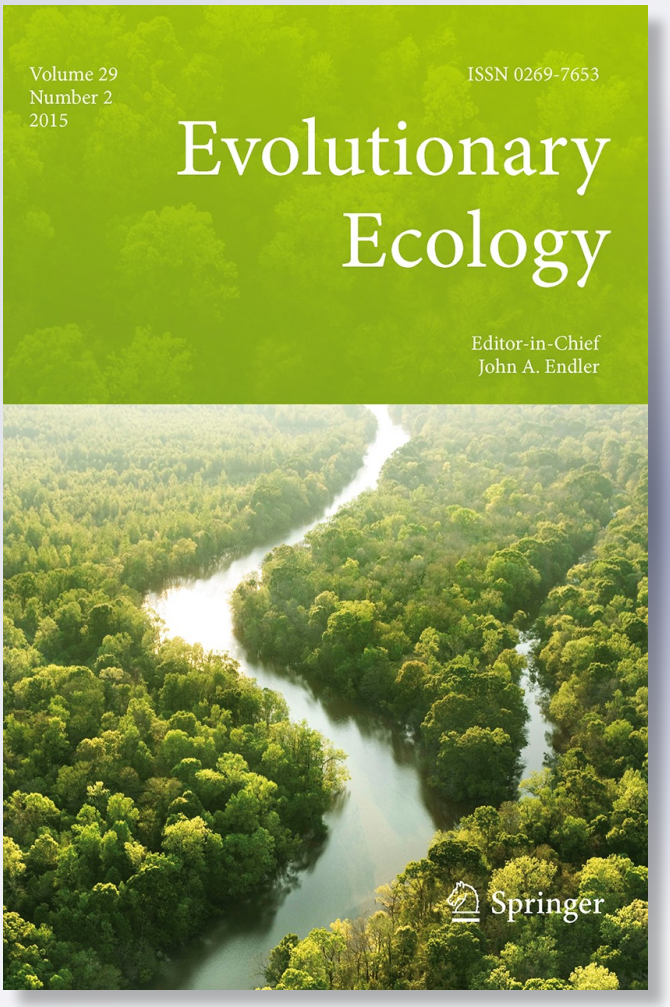

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ORIGINAL PAPER

Strong postzygotic isolation prevents introgression between two hybridizing Neotropical orchids, *Epidendrum denticulatum* and *E. fulgens*

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Abstract Studies on hybrid zones are essential to understand the origin and evolution of reproductive barriers in plants. To achieve this goal, multidisciplinary approaches are often required to investigate the role of multiple reproductive isolation (RI) mechanisms. For *Epidendrum denticulatum* and *E. fulgens*, two Neotropical food-deceptive orchid species, we used molecular, cytogenetic and morphological analyses, experimental crosses and environmental envelope models to assess the strength of the RI and the mechanisms that

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prevent species collapse when hybridization occurs. Based on genetic assignment tests, hybrids between *E. denticulatum* and *E. fulgens* were detected. However, the low frequency of hybrid specimens found, coupled with the high morphological differentiation between parental species, suggested that strong barriers exist to interspecific gene exchange. Indeed, hybrid plants were largely sterile, as determined by meiotic data and crossing experiments. In the hybrid zone studied here, strong postzygotic barriers maintain species integrity, and these RI mechanisms may be also important during early stages of speciation.

Keywords Hybridization meiotic behavior · Orchidaceae · Environmental envelope models · Reproductive isolation · Speciation

Introduction

The analyses of plant hybrid zones provide key information for understanding the strength of species integrity and the mechanisms that modulate gene flow across species barriers (Widmer et al. 2009). Studies on hybrid zones have been crucial to understand the evolution of reproductive isolation (RI) among species because many hybrid combinations can be found in natural populations. Different levels of hybrid fertility have been observed, and the level of fertility influences the degree of interspecific gene flow that leads to introgression (Ståhlberg and Hedrén 2009; Pinheiro et al. 2010; Palma-Silva et al. 2011; Moraes et al. 2013) and, ultimately, species collapse (McKenzie-Gopsill et al. 2012). Even in cases where hybrids are largely sterile, hybridization can provide important information regarding the mechanisms that prevent introgression and, consequently, maintain parental species integrity (Moccia et al. 2007; Marques et al. 2010, 2012). Therefore, the investigation of hybrid zones between recently diverged species may provide important information about the RI mechanisms acting during early stages of speciation (Scopece et al. 2010).

Studies focusing on natural hybrid zones have contributed to the notion that RI among plant species pairs is not associated with a single isolating mechanism but is a consequence of different pre- and post-zygotic barriers, including their complex interactions (Coyne and Orr 2004; Scopece et al. 2007). In addition, the demographic signatures of range contraction/expansion and patterns of intraspecific gene flow (Petit and Excoffier 2009), the time elapsed since the origin of contact zones (Strasburg and Rieseberg 2008) and karyological differences between parental species (Jørgensen et al. 2011; Moraes et al. 2013) all influence the evolutionary outcome of hybridization events.

The evolutionary consequences of plant hybridization are often difficult to predict and are often challenged by the widely held views of 'instant isolation' among species of different ploidy (Coyne and Orr 2004; Soltis et al. 2009). Even in groups of closely related species, variable hybrid zones composed of different frequencies of F_1, F_2 and/or backcrossed individuals have been detected. In Louisiana irises, the fitness of advanced generation hybrids varies across hybrid zones located in different habitats where parental species occur (reviewed by Arnold et al. 2011). In Mediterranean food-deceptive orchids, the prevalence of F_1 hybrids and strong postzygotic barriers between parental species (Moccia et al. 2007; Scopece et al. 2007) were recently rejected as evidence for isolation in some species (Zitari et al. 2012; Pavarese et al. 2013). Thus, information obtained from

hybrid zones composed of different species is crucial to obtain a clearer picture of the evolution of RI in plants. Moreover, the study of plant RI requires the integration of different approaches to detect the main mechanisms influencing interspecific gene flow. Morphological analysis, population genetics, cytogenetics and genome size, environmental envelope models (EEMs) and reproductive experiments are powerful and complementary approaches that have been adopted in current studies investigating plant hybrid zones (Jørgensen et al. 2011; Marques et al. 2010, 2014; Scopece et al. 2013; Vega et al. 2013).

Despite the high levels of plant diversity observed in the Neotropical region, few studies have investigated the evolution of RI in natural hybrid zones (but see Lorenz-Lemke et al. 2006; Cerón-Souza et al. 2010; Palma-Silva et al. 2011; Surget-Groba and Kay 2013; Twyford et al. 2014). The characterization of plant model groups in this region could change this situation because the use and application of multidisciplinary approaches to study different RI barriers would be facilitated. *Epidendrum*, the largest orchid genus in the Neotropics (Hágsater and Soto-Arenas 2005), has recently been used as a biological model system to study the evolution of RI and speciation (Pinheiro and Cozzolino 2013). Together, the extensive morphological diversity observed in this group and the broad variation in chromosome number observed among species, indicate that hybridization events have occurred (Dunsterville 1979; Dressler 1989; Hágsater and Soto-Arenas 2005; Assis et al. 2013). Of particular interest are the species from the subgenus *Amphyglottium* (Pinheiro et al. 2009a; Pessoa et al. 2012), which often have an overlap in flowering periods, shared pollinators and broad sympatric occurrence (Pinheiro and Cozzolino 2013). Moreover, the hybridization between *Epidendrum* species pairs has recently been confirmed with studies adopting the multidisciplinary approaches described above (Pinheiro et al. 2010; Moraes et al. 2013; Vega et al. 2013; Marques et al. 2014). Such studies have revealed wide variation in the frequencies of F_1, F_2 and backcrossed hybrids, even between species with large differences in chromosome number, such as *E. fulgens* ($2n = 24$) and *E. puniceoluteum* ($2n = 56$) (Pinheiro et al. 2010; Moraes et al. 2013), and distinct genome sizes, such as *E. madsenii* (2.69 pg) and *E. falcisepalum* (4.08 pg) (Marques et al. 2014).

In this study, different lines of evidence were used to explore the strength of RI between two hybridizing species, *Epidendrum denticulatum* and *Epidendrum fulgens* (Fig. S1). Both species have been the subject of broad phylogeographical studies (Pinheiro et al. 2011, 2013), and a detailed genetic background is known for the populations examined. Morphometry and assignment tests were used to detect the presence and frequency of hybrids in the sympatric population where the species co-occur. Crossing experiments, anatomical and cytogenetic data were combined to understand the contribution of the different barriers to RI. Furthermore, EEMs were applied to understand how demographic changes in *E. denticulatum* and *E. fulgens* have influenced the patterns of hybridization that were observed. The role of pre- and postzygotic barriers in the maintenance of species integrity and the potential evolutionary mechanisms associated with the maintenance of species barriers are discussed. The main questions of this study are: (1) Do *E. denticulatum* and *E. fulgens* hybridize in the wild, as indicated by the presence of specimens with intermediate morphological characters? (2) If hybridization occurs, what is the genomic composition of the hybrids when assessed using nuclear and plastid DNA markers? (3) What is the strength of RI barriers between parental species, and do hybrids bridge gene flow across ploidy barriers? (4) How have demographic changes such as range expansion/fragmentation influenced the hybridization scenario between *E. denticulatum* and *E. fulgens* in the past, and what are the projections for future hybridization events?

Materials and methods

Study species

Epidendrum denticulatum and *E. fulgens* are perennial terrestrial species that are found with seashore sand dune vegetation. The geographic distributions of the species overlap at Bertioga, where the unique known sympatric population occurs (Fig. 1a). Populations of *E. denticulatum* are also found with the Cerrado (savanna) vegetation inland. *Epidendrum denticulatum* and *E. fulgens* are phylogenetically closely related species, belonging to the Atlantic clade, which is nested within subgenus *Amphyglottium* (Pinheiro et al. 2009a; Pessoa et al. 2012). This group is characterized by *Epidendrum* species pollinated by food-deception, where flowers are visited by several butterfly species, although no reward (nectar) is offered (Pinheiro and Cozzolino 2013). The species are self-compatible, but pollinators are necessary for seed set (Almeida and Figueiredo 2003; Fuhro et al. 2010).

Sampling design and genotyping assays

In addition to the sympatric population at Bertioga, where samples from both species and putative hybrids were collected (Table 1), samples of *E. denticulatum* and *E. fulgens* were obtained from two locations each. Samples were previously genotyped by Pinheiro et al. (2011—*E. fulgens*) and Pinheiro et al. (2013—*E. denticulatum*), and here were used to test for the first time the hypothesis of hybridization between *E. denticulatum* and *E. fulgens*. Seven nuclear loci were analyzed in total (eff26, eff43, eff45, eff48, eff61, epp18, epp86; Pinheiro et al. 2008a, b, 2013). Furthermore, results from five plastid markers were used (Epcp02, Epcp03, Epcp04, Epcp08 and Epcp09; Pinheiro et al. 2009b). Pinheiro et al. (2011, 2013) provide details concerning molecular analyses and genotyping assays.

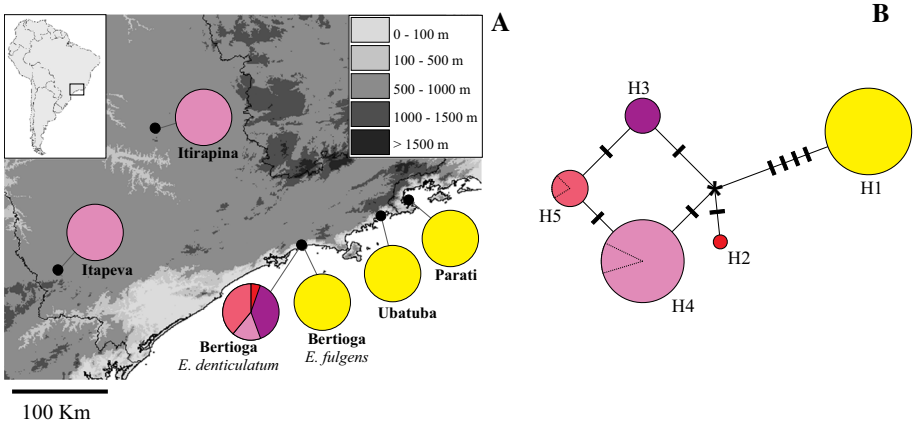

Fig. 1 Map showing the populations sampled of *E. denticulatum* and *E. fulgens* in southeastern Brazil, including the genealogical relationships of the five plastid DNA haplotypes recovered. **a** Pie charts reflect the frequency of occurrence of each haplotype in each population. **b** Statistical parsimony network linking the five haplotypes. Haplotypes are designated by numbers, and circle sizes are proportional to haplotype frequencies. The number of mutations required to explain transitions among haplotypes is indicated by *cross hatches along the lines* connecting the haplotypes. The *dotted lines* inside haplotypes H4 and H5 indicate the frequency of individuals with intermediate levels of nuclear admixture (hybrids)

Table 1 Populations of *Epidendrum denticulatum* and *E. fulgens* studied, including the number of individuals sampled for morphometry (MO), nuclear (NM) and plastid markers (PM)

Population	Species	Vegetation type	Sample Size		
			MO	NM[a]	PM[a]
Paraty	*E. fulgens*	Sand dune scrub vegetation	25	34	16
Ubatuba	*E. fulgens*	Sand dune scrub vegetation	17	18	16
Bertioga	*E. fulgens*	Sand dune scrub vegetation	20	20	15
	E. denticulatum	Sand dune scrub vegetation	18	18	11
	Hybrids	Sand dune scrub vegetation	6	7	7
Itirapina	*E. denticulatum*	Open shrub vegetation	20	23	21
Itapeva	*E. denticulatum*	Open shrub vegetation	9	24	16
Total			115	144	102

[a] Species assignment were based on STRUCTURE results

Nuclear and plastid genetic structuring

Two methods were used to investigate the nuclear genetic admixture between *E. denticulatum* and *E. fulgens*. First, the software HINDEX was used to calculate a molecular hybrid index for each individual (Buerkle 2005). For the treatment here, individuals with a hybrid index of zero are genotypically similar to plants from *E. denticulatum*, whereas plants with a hybrid index of one are similar to *E. fulgens.* Hybrid index values between 0.10 and 0.90 indicate individuals with a hybrid origin. Second, Bayesian assignment analysis implemented by STRUCTURE v. 2.3.3 (Hubisz et al. 2009) was used to assign individuals to genetic clusters (K) and to estimate admixture proportions (q) for each individual. In this study, the $K = 2$ model was used because it was assumed that the two species contributed to the gene pool of the sample. Following Pinheiro et al. (2010), STRUCTURE was used to classify individuals of the two parental species and putative hybrids. Thresholds of $q \geq 0.90$ were used to classify pure individuals of *E. denticulatum*, $q \leq 0.10$ to classify pure individuals of *E. fulgens,* and $0.10 \leq q \leq 0.90$ to classify hybrids. A set of models was chosen in which the individuals had admixed ancestries and correlated allele frequencies. Ten runs using a burn-in of 500,000 and 1,000,000 iterations were performed to check the reliability of the assignment results. The genetic assignment results from HINDEX and STRUCTURE were used to classify the parental and hybrid plants used in crossing experiments and cytogenetic analysis (see below). Results obtained with the program NEWHYBRIDS Anderson and Thompson 2002) were not included due to the low power in detecting parental species and hybrids, as most of the individuals were classified in multiple categories (data not shown). Using the program NETWORK v. 4.5.1.0 (www.fluxus-engineering.com), a haplotype network was constructed using the median-joining (MJ) algorithm (Bandelt et al. 1999) based on plastid DNA to visualize the phylogenetic relationships among haplotypes.

Morphological analyses

Flowers from 115 specimens were collected directly from the field and preserved in 70 % ethanol. Each flower was dissected in the laboratory, and the 16 floral characters (Table S1)

used in previous studies (Pinheiro and Barros 2007; Pessoa et al. 2012) were measured with a digital caliper accurate to the nearest 0.5 mm. Morphological data were analyzed using a discriminant function analysis. The stepwise method, with an F value of 3.84 to enter a variable and an F value of 2.71 to remove it, was applied. The discriminant function was derived using trait measurements from allopatric populations of *E. denticulatum* and *E. fulgens*. The function was used to calculate a morphological assignment, which classified each plant within the sympatric population according to Moccia et al. (2007). The correlation between morphological and genetic assignments was investigated using Spearman's rho method for non-normally distributed data. In addition, principal component analysis (PCA) of morphological data was also conducted as a descriptor of overall morphological differences among taxa in the wild. All analyses were conducted using SPSS 11.0 (SPSS Inc., Chicago, IL, USA).

Crossing experiments

During 2011–2013, controlled pollinations were performed under similar conditions with plants from the Bertioga population (sympatric) at the orchid collection of the Instituto de Botânica (São Paulo, Brazil). Two to four plants were used in each intra- and interspecific experiment (Table 2). In total, 78 flowers from 20 plants were used. Most crosses were performed in both directions such that each plant provided and received pollen. Intraspecific pollinations were used as a control. Fruit formation and seed viability were measured to evaluate the degree of postmating RI among *E. denticulatum*, *E. fulgens* and the hybrids. The seeds were collected from mature capsules and stored at 4 °C. Seed viability rates were determined using the tetrazolium test, following Pinheiro et al. (2010). Samples of 200 seeds per fruit were analyzed with an optical microscope. The percentage of viable seeds was calculated by dividing the number of viable embryos by the total number of embryos scored. Fruit and seed set data were obtained for each treatment and compared using the Mann–Whitney or Kruskal–Wallis test with the SPSS 11.0 software.

Table 2 Estimates of the strength of reproductive isolation between *E. denticulatum* and *E. fulgens*, and between hybrids and parental species, including the number of pollinated flowers, number of fruits produced, the ratio between fruits produced by pollinated flowers (FR/FL), seed viability, and estimates of the strength of different stages of prezygotic (fruit formation) and postzygotic isolation (embryo mortality)

Pollen recipient	Pollen donor	Flower	Fruit	FR/FL	Seed viability (%)	Prezygotic Isolation	Postzygotic Isolation
E. denticulatum (3)	*E. fulgens* (3)	11.00	6.00	54.55	70.69	0.32	0.32
E. fulgens (3)	*E. denticulatum* (3)	11.00	9.00	81.82	65.84		
E. denticulatum (3)	Hybrid (3)	11.00	1.00	9.09	–	0.67	0.99
Hybrid (3)	*E. denticulatum* (3)	7.00	4.00	57.14	0.49		
E. fulgens (3)	Hybrid (3)	7.00	0.00	0.00	–	0.91	0.99
Hybrid (3)	*E. fulgens* (3)	11.00	2.00	18.18	1.10		
E. fulgens (2)	*E. fulgens* (2)	4.00	4.00	100.00	79.06	–	–
E. denticulatum (3)	*E. denticulatum* (3)	3.00	3.00	100.00	82.13	–	–
Hybrid (4)	Hybrid (4)	8.00	0.00	0.00	–	–	–
Hybrid (self-pollinations) (4)		5.00	0.00	0.00	–	–	–

Parentheses enclose the number of specimens used in each hand pollination experiments

–, not estimable

Flowers used in the intra- and inter-specific pollinations, including experiments using hybrids and abscised flowers, were fixed in 50 % FAA for later observation of pollen grain germination and for evaluations of the morphology and growth of the pollen tubes using epifluorescence microscopy (Martin 1959). Following Moraes et al. (2013), flowers were fixed at 10–12 days after pollination. The fixed material was treated with 10 M NaOH at 60 °C for approximately 25 min, washed in distilled water and stained with aniline blue (modified from Martin 1959).

Two postmating isolation indices based on fruit and seed set results were calculated for the interspecific crosses following Scopece et al. (2007), one prezygotic (pollen-stigma incompatibility affecting fruit formation = $RI_{prezygotic}$) and one postzygotic (embryo mortality affecting seed viability = $RI_{postzygotic}$). Because most crosses were bidirectional, reciprocal indices were averaged to provide a mean isolation index for each treatment, where each species was used as both pollen donor and receiver. The pollen-stigma incompatibility isolation index was thus defined as $RI_{prezygotic} = 1 -$ (mean % number of fruits in bidirectional interspecific crosses/mean % number of fruits in bidirectional intraspecific crosses). The embryo mortality isolation index was defined as $RI_{postzygotic} = 1 -$ (mean % seed viability in bidirectional interspecific crosses/mean % seed viability in bidirectional intraspecific crosses). All measures of isolation varied between 0 (no isolation) and 1 (complete isolation).

Cytogenetic analyses

To evaluate the normality of the meiotic process, which culminates with pollen grain production, samples of *E. denticulatum*, *E. fulgens* and the hybrids were analyzed. At least two specimens per locality were sampled, including sympatric and allopatric populations. In total, 18 individuals were analyzed. The results published by Moraes et al. (2013) were used to discuss the meiotic behavior of two specimens of *E. fulgens* from the allopatric population of Paraty. Samples of *E. fulgens* from Ubatuba were not available for cytogenetic analysis.

Floral buds were collected and fixed in ethanol:acetic acid (3:1, v/v) for 24 h at room temperature and stored at −20 °C. To evaluate the meiotic process, pollinia were washed two times in distilled water, digested in 2 % (w/v) cellulase (Onozuka), 20 % (v/v) pectinase (Sigma), and 1 % Driselase (Sigma) at 37 °C for 5 min, and squashed in a drop of 60 % acetic acid. The analyses of all slides evaluated the meiotic normality by the frequency of cells without meiotic abnormalities, e.g., pairing errors, segregation errors, and the presence of micronuclei or telophase II with less/more than four nuclei.

Mitotic analyses were performed using root tips pretreated with 0.002 M 8-hydroxyquinoline (Sigma) for 24 h at 10 °C, fixed in absolute alcohol:glacial acetic acid (3:1, v/v) for 24 h at room temperature and stored at −20 °C. For squash preparations, root tips were washed three times in distilled water, digested in a 2 % (w/v) cellulase (Onozuka), 20 % (v/v) pectinase (Sigma), and 1 % Driselase (Sigma) solution for 30 min at 37 °C, and squashed in a drop of 60 % acetic acid. All slides were examined using Olympus microscopy. Selected cells were photographed with a CCD camera and analyzed using the software Image CellSens (Olympus, Inc.). The images were processed for contrast and brightness uniformity using Adobe Photoshop CS5 (Adobe Systems, Inc.).

Modeling potential distributions for *E. denticulatum* and *E. fulgens*

We obtained 125 *E. denticulatum* and 121 *E. fulgens* locations from our field GPS records and georeferenced herbarium data (Fig. S2A, S2B) extracted from the *species*Link project

(http://splink.cria.org.br). All points were verified with Google Earth to ensure that localities were not used from heavily urbanized areas. To avoid illegal plant collecting, the localities used for EEM analyses are available from the authors upon request.

We modeled the current climate-based distribution using Maxent v.3.3.3 (Phillips et al. 2006). The current model distribution was projected for two late Quaternary scenarios (Last Interglacial (LIG) and Last Glacial Maximum (LGM)), and for future distributions (projected for 2080). Both past scenarios, LIG (~120,000–140,000 years BP; Otto-Bliesner et al. 2006) and LGM (~21,000 years BP; Braconnot et al. 2007) consider the CCSM3 model (Collins et al. 2006). The future climate predictors were derived from the general circulation model (CCCMA: CGCM2) for 2080, under the IPCC emission scenario (A2a) for predicting future distributions (Ramirez and Jarvis 2008). Scenario A2a assumes that changes in temperature and precipitation will be more intense. We used 19 bioclimatic variables derived from monthly temperature and rainfall following the Bioclim scheme (Hijmans et al. 2005; http://www.worldclim.org/), altitude (SRTM V2) and geology (Table S2). We used the three geological variables that were derived from Schenk et al. (1999), major geological types (sedimentary, igneous and metamorphic rocks), eras and geological age (Table S2). Major geological types and eras were categorical and geological age was continuous. All variables were scaled to the same spatial resolution of 30 arc seconds (ca. 1 km^2). Highly correlated variables were removed ($r > 0.7$), and jackknifing was used to estimate variable importance. The final models were obtained by only considering variables with contributions to an AUC higher than 75 %. Ten model replicates were run for each one of the presence-only methods, with 75 % of the occurrences used for calibration and different subsets (25 %) used for validation. The mean AUC was used to assess the performance of the models (Fielding and Bell 1997), where 1.0 was the maximum prediction and 0.5 suggested a random prediction. Past and future distributions were estimated by assuming that the current relationships between climate and distribution are maintained.

We used Shoener's *D* (Schoener 1968) to evaluate the degree of niche overlap between *E. denticulatum* and *E. fulgens* through time as implemented in the program ENMtools (v.1.4.4) (Warren et al. 2010) to measure EEM similarity. The *D* compares probabilities of distributions for species over geographic space and time from 0 to 1.0, where 0 indicates that the EEMs of two species have no overlap and 1.0 indicates that the EEMs are identical.

Results

Plastid and nuclear genetic structure

The analyses performed in HINDEX and STRUCTURE consistently identified pure *E. denticulatum* and *E. fulgens* specimens in all populations (threshold values $\geq$0.90 or $\leq$0.10, respectively). Nine individuals from Bertioga were identified as hybrids by HINDEX (Fig. 2a), while seven plants from the Bertioga population and one from the Itirapina population showed signs of admixed ancestry using STRUCTURE (Fig. 2b), indicating hybrid ancestry. The analyses of five plastid loci recovered a total of four haplotypes for *E. denticulatum* and one haplotype for *E. fulgens* (Fig. 1b). More than one haplotype was observed only in the *E. denticulatum* population from Bertioga. Haplotype sharing between species was not observed. The network showed a clear differentiation between *E. denticulatum* and *E. fulgens* haplotypes, which are separated by four mutational steps (Fig. 1b). Haplotype H1 and H4 were the most frequent, occurring in all populations of *E.*

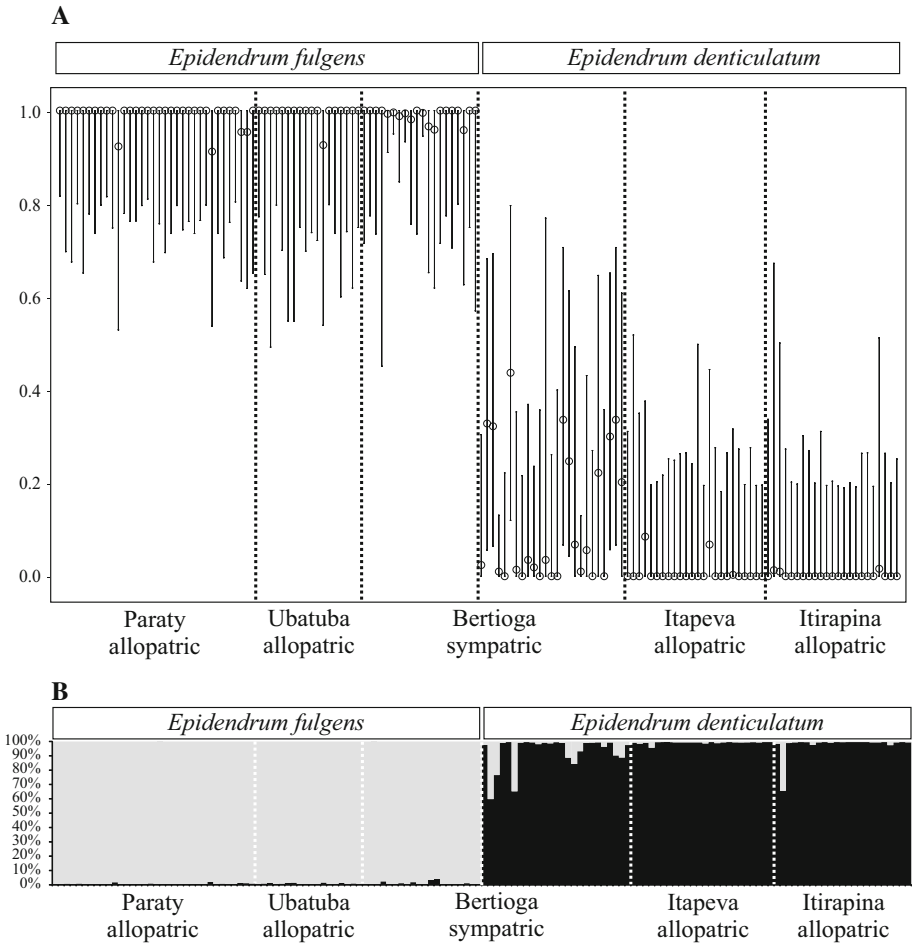

Fig. 2 Genetic assignment results obtained with HINDEX (**a**) and STRUCTURE (**b**) for populations of *E. denticulatum* and *E. fulgens*, including the sympatric population Bertioga. **a** Molecular hybrid indices (±SD) for individuals of *E. denticulatum* (low molecular hybrid scores), *E. fulgens* (high molecular hybrid scores) and hybrids (molecular hybrid scores between 0.1 and 0.9); **b** posterior probabilities (q) for *E. denticulatum*, *E. fulgens* and hybrids. Each *vertical bar* represents an individual. The proportion of *color* in each *bar* represents an individual's assignment probability, which correspond to *E. denticulatum* (*black*) and *E. fulgens* (*gray*). See Fig. 1 for details of geographical position of each locality

denticulatum (H4) and *E. fulgens* (H1). Haplotypes H2, H3 and H5 were restricted to the Bertioga population.

Morphological variation in parental species and hybrid individuals

The discriminant function analysis found significant morphological differences between *E. denticulatum* and *E. fulgens* (Wilks' Lambda = 0.033, $P < 0.001$). Strong correlation was found between the morphological and the molecular assignment values (correlation coefficient = 0.829, $P < 0.01$). The PCA of 115 specimens indicated that the first two

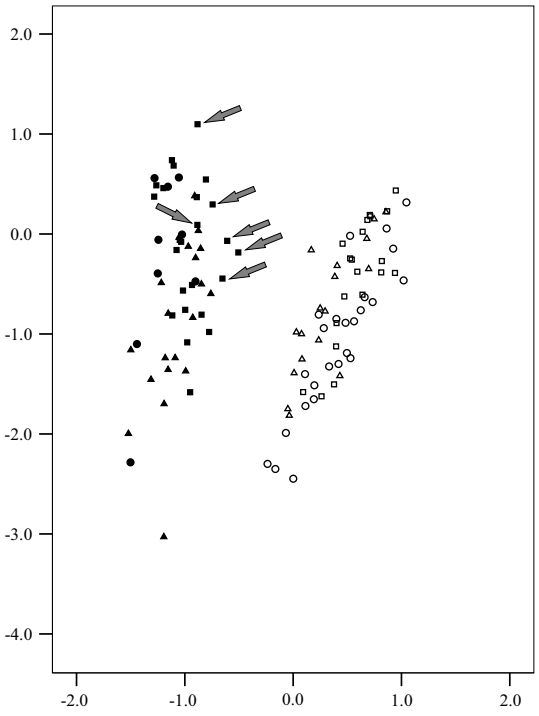

Fig. 3 Principal coordinate analysis of the sixteen floral morphological measurements that were scored for 115 individuals of *E. denticulatum* (*filled symbols*), *E. fulgens* (*empty symbols*) and hybrids (*gray arrows*). The first two derived factors accounted for 58.3 and 12.2 % of the observed variance between individuals. Population codes: *filled circles* Itapeva, *filled triangles* Itirapina, *filled squares* Bertioga (*E. denticulatum*), *empty squares* Bertioga (*E. fulgens*), *empty triangles* Ubatuba, *empty cycles* Paraty. The identification of hybrid plants was based on STRUCTURE results

derived factors accounted for 70.5 % of the observed variance among all populations analyzed. The scatterplot of the first two components showed a clear separation between individuals from the two parental species, primarily associated with the first principal component (*x*-axis). Separation among different populations within each species was not clear, and only a gradient of variation was observed associated with the second principal component (*y*-axis). The plants of hybrid origin were plotted among *E. denticulatum* individuals (Fig. 3).

Artificial crosses

All intraspecific pollinations produced fruits, and seed viability ranged from 78.4 to 85.1 % in *E. denticulatum* and from 73.4 to 85.9 % in *E. fulgens* (Table 2). Interspecific crosses also produced fruits in most experiments, but seed viability (48.02–94.45 %) was significantly lower when compared with intraspecific seed viability ($U = 18.000$, $P < 0.01$). A significant decrease in fruit and seed set was also found when hybrids were included in the crossing experiments (Table 2). Fruit and seed set in intraspecific crosses was significantly higher than crosses in which hybrids received or donated pollen to *E. denticulatum* or *E. fulgens* (fruit set: $U = 24.500$, $P < 0.001$; seed viability: $U = 0.000$, $P < 0.01$). For the crosses between hybrids and parental species, only one fruit was produced when a hybrid plant acted as pollen donor. Self-pollinations performed with hybrids and crosses between different hybrid individuals did not set fruits.

Pollen germination and pollen tube growth was observed in flowers from all crossing experiments (Fig. S3A-F). However, the hybrid pollen grains showed a twisted appearance

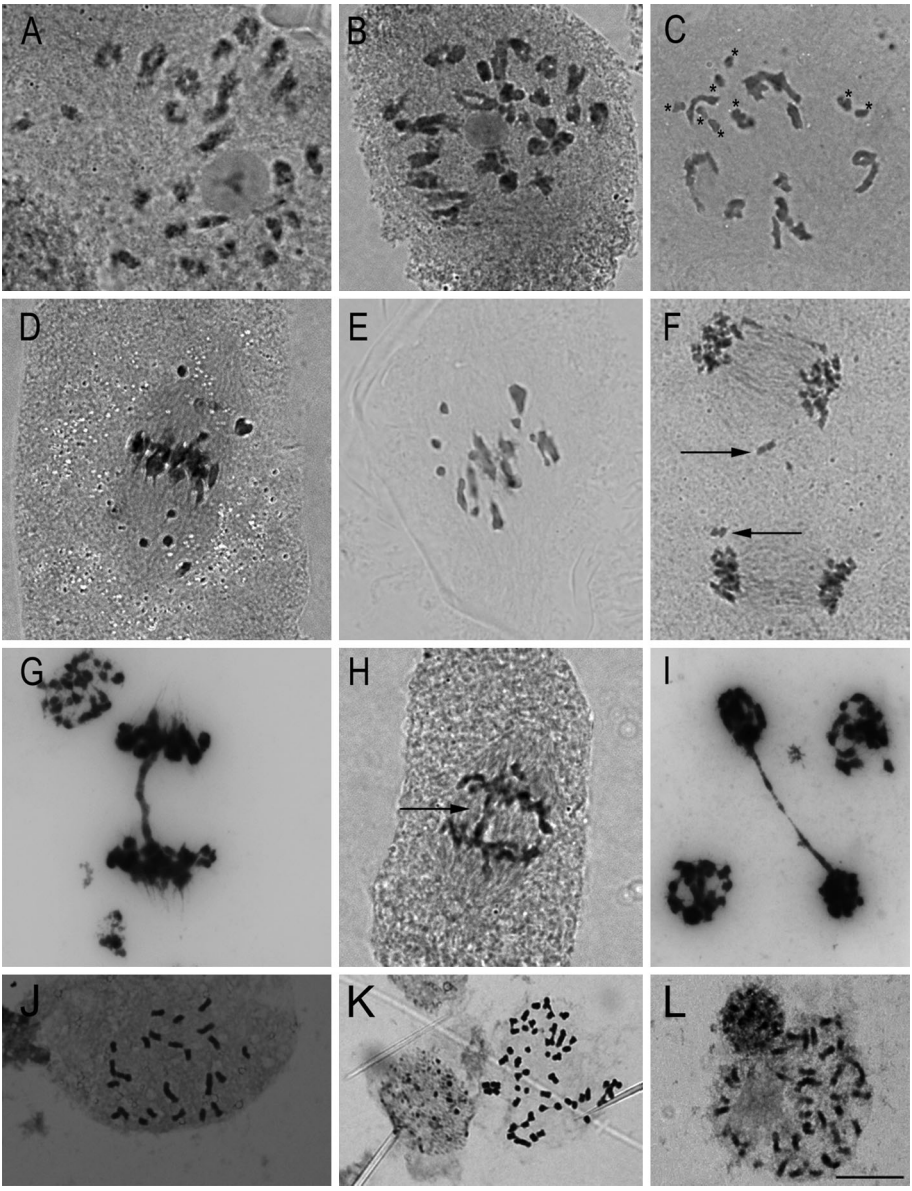

Fig. 4 Meiotic process and mitotic counts in *E. fulgens*, *E. denticulatum* and hybrids. Normal meiosis in *E. denticulatum* with $n = 26$ (**a**, **b**); hybrid meiosis with $n = 20$ (**c**), presenting 8 univalents (*asterisks*); metaphase I with univalents non-orientated (**d**, **e**); fragments in telophase II (**f**); bridge in Anaphase I (**g**, **h**); bridge and fragment in Telophase II (**i**). Chromosome counts are shown for *E. fulgens* with $2n = 24$ (**j**), *E. denticulatum* with $2n = 52$ (**k**) and hybrid with $2n = 40$ (**l**). *Scale bar* in **l** is 10 μm

when they reached the initial portion of the style and displayed an irregular trajectory and excessive deposition of callose material on the pollen tube walls (Fig. S3E-F). This pattern was observed when hybrids acted as pollen donors to *E. denticulatum*, *E. fulgens* and other hybrid plants. In the crosses where *E. denticulatum* and *E. fulgens* acted as pollen donors,

pollen grains germinated in large quantities, their pollen tubes grew straight and had callose plug deposits regularly spaced along their length, and the pollen tubes reached the base of the column and ovary (Fig. S3A-D).

Reproductive isolation indices were calculated for 58 interspecific crosses (Table 2). The RI between *E. denticulatum* and *E. fulgens* was incomplete, and the same values of RI indices were observed for fruit set and seed viability (0.32). In contrast, almost complete RI indices (RI = 0.99) were observed between parental species and hybrids, considering either fruit set (no fruit formation in 29 out of 36 crosses) or seed viability (0.25 % to 0.55 % of seed viability).

Meiotic and mitotic analyses

Individuals of *E. fulgens* from allopatric and sympatric populations presented $n = 12$, and low numbers of abnormal cells were observed in meiosis (0.5–1.1 %, Fig. 4; Table 3). Samples of *E. denticulatum* presented $n = 26$ in both sympatric and allopatric populations, and the meiotic abnormalities were also low (0.7–6.5 %). Hybrid plants presented $n = 20$ and had higher levels of meiotic abnormalities, ranging from 19.4 to 88.5 %. The lagging chromosomes in metaphase I and anaphase I were the most common abnormalities, in addition to bridges and univalency. In accordance with the meiotic counts, mitotic metaphases from *E. fulgens* and *E. denticulatum* presented, respectively, $2n = 24$ and $2n = 52$ (Table 3). The hybrid specimens presented $2n = 40$ and were the same individuals where meiotic irregularities were detected. In addition, polyploidization was not observed in any hybrid plant.

Environmental envelope models for *E. denticulatum* and *E. fulgens*

Estimated distributions of suitable habitats were successfully obtained for *E. denticulatum* and *E. fulgens* for the LIG, LGM, current and future climatic conditions. The EEM results showed high performance as indicated by the AUC values (*E. denticulatum* AUC = 0.9302; *E. fulgens* AUC = 0.9012). The distribution of suitable environments predicted for *E. fulgens* was more stable among different climatic conditions and showed fewer geographic changes than the distribution models of *E. denticulatum*. During the LIG (Fig. S2C), the predicted distribution of suitable habitats was fragmented and associated with the coast for both species, and inland only for *E. denticulatum*. It was during the LIG period that the potential distribution of favorable areas for both species largely overlapped

Table 3 Meiotic normality and chromosome number of *E. denticulatum*, *E. fulgens* and hybrids

Population	Species	N	Mean abnormal meiosis (%)	Haploid/diploid chromosome number
Paraty[a]	*E. fulgens*	2	0.45	12/24
Ubatuba	*E. fulgens*	–	–	–
Bertioga	*E. fulgens*	2	1.1	12/24
	E. denticulatum	6	5.45	26/52
	Hybrids	4	45.75	20/40
Itirapina	*E. denticulatum*	4	1.68	26/52
Itapeva	*E. denticulatum*	2	3.2	26/52

[a] Data from Moraes et al. (2013)

(Table S3), approximately 300 km south of Bertioga (Fig. S2C). During the LGM period, the models indicated a contraction in the distribution of suitable environmental conditions for both species, followed by a decrease in habitat overlap (Fig. S2D). From the LIG to the LGM, the intersection of environmental suitable habitats of *E. denticulatum* and *E. fulgens* moved to the north, close to the current location of the hybrid zone in Bertioga. The EEM for the LGM (Fig. S2D) predicted a small increase in the distribution of favorable areas for *E. fulgens*, whereas the habitat suitability of *E. denticulatum* decreased along the seashore and inland. The models for current conditions (Fig. S2E) showed that habitat conditions along the coast were favorable for *E. fulgens*, while the habitat suitability for *E. denticulatum* was broadly distributed inland, with a more fragmented distribution close to the seashore. When projecting the EEMs to the near future (2080) (Fig. S2F), the results indicated the potential persistence of favorable habitats along the coast for *E. fulgens*, but a contrasting pattern for *E. denticulatum* habitats, which almost disappeared in the coastal region and occurred mainly inland.

The evolution of the predicted niche overlap between the species showed a maximum overlap during the LIG, a significant reduction during the LGM, but with a slight increase under present climate conditions. However, the most dramatic reduction of spatial overlap between suitable habitats for *E. denticulatum* and *E. fulgens* was projected for the near future (Table S3). Consequently, the climatic changes predicted to occur in less than a century will potentially decrease the habitat overlap between both species.

Discussion

The different lines of evidence confirmed the hybridization between *E. denticulatum* and *E. fulgens*. The admixed individuals occurring in the sympatric population at Bertioga were identified by nuclear markers (Fig. 2a, b), and the plastid data set revealed that *E. denticulatum* acted as the female parent in the hybridization events (Fig. 1b). Indeed, hybrids were morphologically more similar to *E. denticulatum* than to *E. fulgens*. Fruits and viable seeds were formed in the interspecific crosses, but low levels of fruit formation and seed viability were found in crosses where admixed individuals acted as pollen donors or receptors, confirming their hybrid origin. The different chromosome numbers found between parental species (*E. denticulatum*, $2n = 52$ and *E. fulgens*, $2n = 24$) did not preclude the formation of hybrids, but most likely affected the fertility of the offspring. Meiotic abnormalities were found in the hybrid plants, and pairing problems were the most common abnormalities found among the hybrids. Surprisingly, molecular markers did not provide any evidence of introgression and interspecific gene flow in this hybrid zone, although this has been observed for other studies investigating *Epidendrum* species occurring in sympatry (Pinheiro et al. 2010; Vega et al. 2013; Marques et al. 2014). Consequently, the study of different hybrid zones added important information about the strength and direction of reproductive barriers between phylogenetically closely related species. Our multidisciplinary approach also provided significant information about the potential ecological scenarios and genetic mechanisms underlying the evolution of RI during early stages of speciation.

Morphological variation and species integrity

Extensive morphological variation was observed within each species, which has been noted in orchid species pollinated with a food-deceptive strategy (Jersáková et al. 2006;

Ackerman et al. 2011). The genus *Epidendrum* is primarily pollinated by deceit and is well known for its morphological variability, which can challenge the delimitation and taxonomic recognition of many species groups (Hágsater 1984; Hágsater and Soto-Arenas 2005). An increase in the variability of a floral trait would decrease pollinator ability to learn to avoid the nectarless flowers, and thereby increase their visitation rate. However, this hypothesis is not supported by currently available studies (reviewed by Juillet and Scopece 2010). The floral variability observed in food-deceptive species, particularly in *Epidendrum*, might be a consequence of relaxed selection by pollinators, which do not discriminate for specific floral characters. Indeed, *E. denticulatum* and *E. fulgens* are pollinated by many pollinator species (Almeida and Figueiredo 2003; Fuhro et al. 2010), a pattern commonly observed in other food-deceptive species (Cozzolino and Scopece 2008).

Large morphological differences were found between *E. denticulatum* and *E. fulgens*, and multivariate analyses clearly identified morphological discontinuities between the species, even in the sympatric population at Bertioga (Fig. 3). The selection for different pollinator species can maintain floral divergence even in sympatry with assortative mating (Fulton and Hodges 1999). For example, the low frequency of hybrids found in this hybrid zone suggested that pollinator sharing between these species might be rare. The lower hybrid viability, such as low germination of hybrid seeds and development problems for mature plants, could also explain the low frequency of hybrids in nature. In contrast, in other *Epidendrum* hybrid zones, where a large number of hybrid plants were found, parental species identification was difficult because of gradients of morphological variation between the parental species and the hybrids (Pinheiro et al. 2010; Vega et al. 2013; Marques et al. 2014). Field experiments and observations of pollinators might clarify the occurrence of assortative mating between *E. denticulatum* and *E. fulgens* in the Bertioga sympatric population because pollinator-mediated selection may vary through time and space according to local variations in pollinator assemblages (Moccia et al. 2007; Moré et al. 2012). Furthermore, many studies have shown that floral traits alone were not sufficient to maintain species differences in sympatry (Natalis and Wesselingh 2013), suggesting that postmating barriers were also important isolating mechanisms in such circumstances. Because the meiotic process in the hybrid individuals was disrupted with huge abnormalities, this could present a strong postmating barrier and prevent the sexual reproduction of the hybrids and the backcrossing of hybrids with parental species.

Intrinsic postmating barriers between *E. denticulatum* and *E. fulgens*

Strong barriers to gene exchange between *E. denticulatum* and *E. fulgens* were found in the sympatric population of Bertioga (Table 2). The ease with which F_1 hybrid seeds were produced in the manual crosses did not occur in crossing experiments that included hybrid plants. Incomplete RI barriers were found in the crosses between *E. denticulatum* and *E. fulgens*, suggesting that hybrids were easily produced by artificial pollination experiments. In contrast, the strength of RI approached 1.0 in most experiments where hybrids acted as pollen donors and receptors to parental species (Table 2), indicating that hybrid sterility was the main barrier to prevent introgression and interspecific gene flow.

Results obtained for different plant groups (Moyle et al. 2004; Scopece et al. 2008; Jewell et al. 2012) have shown that the strength of postzygotic barriers increased with increasing genetic distance, which suggested slow evolution of postzygotic barriers. According to Scopece et al. (2008), later postzygotic isolation mechanisms, such as hybrid sterility, evolved faster than early postzygotic isolation mechanisms, such as embryo

mortality. The prevalence of late postzygotic isolation mechanisms between *E. denticulatum* and *E. fulgens* was in agreement with the close phylogenetic relationship observed for these species (Pinheiro et al. 2009a; Pessoa et al. 2012). In general, premating and postmating barriers have been detected in plant hybrid zones within the Neotropics. The role of reinforcement and prezygotic barriers preventing hybrid formation was rarely documented for Neotropical plants (but see Surget-Groba and Kay 2013). Multiple premating (flowering phenology, pollinator isolation) and postmating barriers (genic incompatibilities) were found in *Pitcairnia* (Bromeliaceae) hybrid zones, despite the extensive haplotype sharing between parental species (Palma-Silva et al. 2011). On the other hand, postzygotic barriers were more commonly reported in the literature, as observed for *E. denticulatum* and *E. fulgens*. Hybrid sterility due to genome size differences (Twyford et al. 2014), habitat selection (Cerón-Souza et al. 2010; Pinheiro et al. 2010) and genic incompatibilities (Caddah et al. 2013) are potential postzygotic barriers preventing parental species collapse in these hybrid zones.

The gradual evolution of postzygotic isolation barriers can be associated with genetic incompatibilities and/or chromosomal mutations (changes in the structure or number of chromosomes). Inviable hybrids and hybrid sterility are frequently attributed to the fixation of incompatible loci, as predicted by the Bateson–Dobzhansky–Muller (BDM) classic model of genic incompatibilities (Coyne and Orr 2004). BDM incompatibilities may result from a simple negative genetic interaction among a few nuclear loci (reviewed in Bomblies and Weigel 2007). The gradual accumulation of BDM incompatibilities might need more time to arise because of its multigenic origin, and thus would be expected to act at earlier stages of postzygotic isolation, such as embryo mortality (Orr and Turelli 2001). The later stages of postzygotic isolation, as observed between *E. denticulatum* and *E. fulgens*, are generally affected by chromosomal rearrangements (Stebbins 1971; Rieseberg 2001), which have a strong influence on hybrid seeds and pollen viabilities.

Extensive meiotic abnormalities were observed in the hybrid plants (Fig. 4), suggesting the role of chromosomal rearrangements in hybrid sterility. The irregular pollen tube growth (Fig. S3E-F) that was observed contributed to the high levels of hybrid male sterility, and when hybrids acted as pollen donors in crossing experiments, fruit formation was inhibited and seed viability was decreased (Table 2). Several cases of genome duplication and the consequent restoration of hybrid fertility are reported in the literature (reviewed by Soltis et al. 2010; Abbott et al. 2013). However, such an event is common in homoploid hybrids formed by two closely related parental species with similar karyotypes (Buerkle et al. 2000) but not in hybrids formed from parental species with contrasting karyotypes, such as *E. fulgens* and *E. denticulatum*. Indeed, polyploidization was not observed in any hybrid plant. The contrasting karyotypes prevented chromosome pairing during hybrid meiosis I and caused the observed low fertility. In addition, the hybrids presented an ascending aneuploidy variation ($2n = 40$) compared with the expected chromosome number ($2n = 38$), most likely a consequence of unbalanced gametes, which have been found in *Epidendrum* species (Da Conceição et al. 2006; Moraes et al. 2013).

The important role of chromosomal changes in plant RI and speciation is uncontroversial (Rieseberg 2001; Levin 2002; Rieseberg and Willis 2007). Chromosomal rearrangements preclude normal chromosome pairing and cause the absence of recombination and irregular segregation during meiosis in hybrid progeny, resulting in unfit gametes that reduce F_1 hybrid fertility (Stebbins 1971; Faria and Navarro 2010). Many genes are expressed in the male gametes of plants, and pollen carrying chromosomal irregularities abort more often than female gametes, which tend to be more tolerant of chromosomal rearrangements (Tanksley et al. 1981; Rieseberg 2001). The karyotype differences

observed between *E. denticulatum* and *E. fulgens* were thus most likely to cause the late postzygotic RI that was observed, and these differences will likely play an important role in maintaining species boundaries in secondary contact zones, as has been observed for other plant species (Husband and Sabara 2004; Moccia et al. 2007; Buggs et al. 2011; Castro et al. 2011).

Demographic changes shaping hybrid zone formation

The presence of only *E. denticulatum* haplotypes in the hybrids (Fig. 1) indicated that hybridization occurred in only one direction, where *E. fulgens* acted primarily as a pollen donor. Intrinsic barriers precluding *E. fulgens* to be the female parent in hybrid formation, as hybrid inviability, may explain this pattern. However, since crossing experiments returned similar patterns of compatibility between both parental species (Table 2), the potential inviability of hybrid plants remains to be tested using field experiments. Different frequencies of parental individuals in the Bertioga hybrid zone could be an alternative explanation for the prevalence of *E. denticulatum* haplotypes in hybrid plants. When one of the parental species occurs at a higher frequency, the rarer species will necessarily behave more often as a pollen recipient, and thus as the female parent in hybrid formation (Rieseberg 1997; Moccia et al. 2007; Lepais et al. 2009). In this scenario, the alleles that originated from the interspecific gene exchange tend to be purged from the genome if the levels of intraspecific gene flow are high, preventing introgression (Petit and Excoffier 2009).

Results obtained by EEM analyses provide important information regarding the geographic changes of suitable habitats for *E. denticulatum* and *E. fulgens*. The EEM analyses predicted a more stable distribution of suitable habitats for *E. fulgens*, than for *E. denticulatum*. The potential long-term persistence of environmentally suitable conditions for *E. fulgens* along the coast during historic climatic oscillations (Fig. S2C-S2F) is in agreement with previous phylogeographic results (Pinheiro et al. 2011). On the other hand, contrasting results were obtained for *E. denticulatum*. Marked changes in the distribution of suitable habitats were predicted from the LIG to the present, confirming previous phylogeographic results that indicated bottlenecks and range contraction mainly in the populations of Itirapina, Itapeva and Bertioga (Pinheiro et al. 2013). Differences in the suitable areas for the occurrence of *E. denticulatum* and *E. fulgens* were also obtained by the future predictions of the EEM analysis, which projected the disappearance of *E. denticulatum* habitats from the coastal region and the persistence of *E. fulgens* habitats in the Bertioga region (Fig. S2F). Overlapping suitable habitats for both species will be a potentially rare situation in the near future, and potential changes in hybridization patterns between *E. denticulatum* and *E. fulgens* may occur (Table S3).

The absence of backcross hybrids and the prevalence of F_1 individuals in a sympatric population might indicate a hybrid zone with a recent origin, in which there has not been sufficient time to produce advanced generation hybrids (Milne et al. 2003; Chung et al. 2005). Accordingly, our results support this hypothesis because the hybrid zone between *E. denticulatum* and *E. fulgens* was probably composed of F_1 plants. However, the EEM results predicted a potential overlap in parental species distribution since the LIG (Fig. S2C-S2E), suggesting that previous hybridization events might have occurred between *E. denticulatum* and *E. fulgens*. On the other hand, Moccia et al. (2007) found that the occurrence of F_1 individuals was independent of the age of the different hybrid zones studied. Consequently, the most likely scenario for the preponderance of first-generation hybrids is the existence of strong pre- and/or postmating barriers that limited the formation

and the persistence of advanced generation hybrids. Therefore, the selection for different pollinators, strong hybrid sterility because of meiotic abnormalities, and different demographic histories are most likely the most important RI mechanisms that limit the introgression and gene flow across the species barriers between *E. denticulatum* and *E. fulgens*. Due to the close phylogenetic position of both species (Pinheiro et al. 2009a; Pessoa et al. 2012), the RI mechanisms preventing interspecific gene exchange may have played an important role during the early steps of speciation.

Conclusions

Different pre- and postzygotic barriers and their potentially complex interactions play crucial roles in the maintenance of RI in plants (Coyne and Orr 2004; Rieseberg and Willis 2007). Thus, barriers to gene exchange may differ in strength and direction and vary within and among plant species pairs. Moreover, plants have provided illustrative examples showing how RI barriers may vary in number and intensity between closely related species pairs (Bleeker and Matthies 2005; Moccia et al. 2007; Marques et al. 2012; Zitari et al. 2012; Arnold et al. 2011). In *Epidendrum*, extensive levels of introgression have been reported in previous studies (Pinheiro et al. 2010; Vega et al. 2013; Marques et al. 2014), which contrasts with the strong postzygotic barriers observed in this study. Hybrid sterility because of meiotic abnormalities was the most efficient barrier preventing gene exchange between *E. denticulatum* and *E. fulgens*. However, complementary isolating mechanisms such as selection for different pollinators and different demographic histories of the parental species may contribute to the maintenance of species integrity. Progress toward understanding the evolution and the importance of different barriers should focus on the details of RI mechanisms in groups of species where this knowledge is still absent. The evidence for multiple and different RI mechanisms acting between closely related plant species will bring novel insights and create interesting venues for future research.

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