RESEARCH ARTICLE

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Strong but permeable barriers to gene exchange between sister species of *Epidendrum*[1]

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PREMISE OF THE STUDY: The investigation of reproductive barriers between sister species can provide insights into how new lineages arise, and how species integrity is maintained in the face of interspecific gene flow. Different pre- and postzygotic barriers can limit interspecific gene exchange in sympatric populations, and different sources of evidence are often required to investigate the role of multiple reproductive isolation (RI) mechanisms.

METHODS: We tested the hypothesis of hybridization and potential introgression between *Epidendrum secundum* and *Epidendrum xanthinum*, two Neotropical food-deceptive orchid species, using nuclear and plastid microsatellites, experimental crosses, pollen tube growth observations, and genome size estimates.

KEY RESULTS: A large number of hybrids between *E. secundum* and *E. xanthinum* were detected, suggesting weak premating barriers. The low fertility of hybrid plants and the absence of haplotype sharing between parental species indicated strong postmating barriers, reducing interspecific gene exchange and the development of advanced generation hybrids. Despite the strength of reproductive barriers, fertile seeds were produced in some backcrossing experiments, and the existence of interspecific gene exchange could not be excluded.

CONCLUSIONS: Strong but permeable barriers were found between *E. secundum* and *E. xanthinum*. Indeed, haplotype sharing was not detected between parental species, suggesting that introgression is limited by a combination of genic incompatibilities, including negative cytonuclear interactions. Most taxonomic uncertainties in this group were potentially influenced by incomplete RI barriers between species, which mainly occurred sympatrically.

KEY WORDS assignment tests; cytonuclear incompatibility; hybridization; introgression; Orchidaceae; pollen–pistil interaction; postzygotic barriers; reproductive isolation; speciation; species delimitation

Since the seminal works of Dobzhansky (1937) and Mayr (1942), the definition of the term "species" has been closely connected to the evolution of reproductive isolation (RI). These authors considered the speciation process per se as a product of the gradual accumulation of reproductive barriers, leading to the formation of new species. Thus, the study of RI mechanisms between closely related species has been a common goal for most researchers interested in the origin and maintenance of incipient species. The bridge between systematists, taxonomists, and evolutionary researchers lies in the correspondence between the strength of reproductive isolation and taxonomic species delimitation. Almost 70% of species recognized by taxonomists show some degree of RI (Rieseberg et al., 2006). Thus, multidisciplinary approaches employed to investigate the mechanisms involved in species formation and cohesion, such as those highlighted by integrative taxonomy (Padial et al., 2010), should also include measures of RI. In this context, plant hybrid zones have offered important insights into the evolution of RI in nonmodel species. The need to better understand the evolutionary outcomes of natural hybridization and the dynamics of reproductive barriers in nonmodel organisms have been highlighted in recent reviews (Abbott et al., 2013; Baack et al., 2015). Instead of viewing hybrid zones as inconvenient sites where static species definitions are hard to fit, sympatric populations where different species hybridize are crucial to understand the origin and maintenance of species integrity (Harrison, 1993).

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The strength of RI, and therefore, the genetic architecture of hybrid zones may vary considerably (Baack et al., 2015). In some cases hybridization occurs, but it is restricted to the F1 generation and is often associated with the sterility of hybrids that are not able to act as a link to interspecific gene exchange (Pinheiro et al., 2015; Twyford et al., 2015). In other cases, hybrids are partially or fully fertile and introgression takes place, resulting in advanced generation hybrids and blurring parental species boundaries (Jacquemyn et al., 2012; De La Torre et al., 2014). Because multiple pre- and postmating barriers can act in concert, preventing or limiting interspecific gene exchange, the study of hybrid zones often adopts multidisciplinary approaches (e.g., Scopece et al., 2013; Vega et al., 2013). These studies have shown that when premating barriers are weak, different intrinsic postmating barriers—such as pollen–stigma incompatibility, fruit abortion, hybrid inviability, and hybrid sterility—may prevent species admixture (Cozzolino and Scopece, 2008). In plants, intrinsic pre- and postzygotic isolation mechanisms are frequently attributed to negative cytonuclear or gametophyte–sporophyte interactions, which follow the Bateson-Dobzhansky-Muller (BDM) classic model of genic incompatibility (Turelli and Moyle, 2007; Scopece et al., 2008; Palma-Silva et al., 2011). Other studies also identified extrinsic barriers to gene exchange, with low hybrid performance being the most common (Ramsey et al., 2003; Roda et al., 2013; Ren et al., 2014).

A creative role of hybridization has long been recognized in orchids because of the ornamental value of artificial hybrids and derived varieties (Arditti, 1984). Recent studies have added surprising complexity and detail to the previously simplistic views of the main RI barriers in orchids (i.e., premating isolation only; see van der Pijl and Dodson, 1966). Premating isolation mechanisms associated with specific pollinators are certainly of great importance in some groups, for example, in sexual-deceptive orchids (Scopece et al., 2010) and species pollinated by Meliponinae bees (Pansarin et al., 2006) and flies (Borba and Semir, 2001). In contrast, multiple postmating barriers play an important role in orchid species integrity in Mediterranean food-deceptive species, which typically have lower pollinator specificity (Scopece et al., 2008; De Hert et al., 2011).

Different patterns of RI do not appear to be restricted to particular orchid groups, because hybrid zones with diverse genetic architectures and extensive levels of genome permeability were observed within the same genus, such as *Dactylorhiza*, *Orchis*, *Anacamptis*, and *Epidendrum*. Sympatric species within this genus often show permeable barriers to gene exchange at different degrees, in which hybrids show full or partial fertility (Pinheiro et al., 2010; Vega et al., 2013; Marques et al., 2014) or complete sterility (Pinheiro et al., 2015). Another remarkable feature of *Epidendrum* hybrid zones is the apparent independence between differences in the chromosome number and ploidy level of the parental species, and patterns of hybridization (Pinheiro et al., 2010; Pinheiro and Cozzolino, 2013). Indeed, chromosomal barriers, which are an effective mechanism for preventing gene exchange between plant species (Abbott et al., 2013; Baack et al., 2015), do not appear to preclude admixture and introgression between different ploidy levels in *Epidendrum* species (Pinheiro et al., 2010; Vega et al., 2013). Because of its multiplicity of potential reproductive barriers, *Epidendrum* has been proposed as a model species to investigate the evolution of reproductive barriers in Neotropical plants (Pinheiro and Cozzolino, 2013).

In this study, the strength of RI between two hybridizing sister species, *E. secundum* and *E. xanthinum* (Fig. 1) of the subgenus *Amphyglottium,* was investigated. Both species co-occur along the Serra dos Órgãos mountain chain (Rio de Janeiro, Brazil) in areas that are difficult to access. The species are characterized by different flower colors and ploidy levels (*E. secundum*, pink flowers, $2n = 56$; *E. xanthinum*, yellow flowers, $2n = 28$; Assis, 2013). However, large numbers of plants showing intermediate flower characters have been observed in sympatric populations (Fig. 1), puzzling species delimitation, and suggesting that hybridization may have occurred. Nuclear and plastid microsatellites were used to examine the genetic background of *E. secundum*, *E. xanthinum*, and the putative hybrids, and assignment tests were used to detect the presence and frequency of hybrids in the sympatric population where these species co-occur. Artificial crossing experiments and pollen tube growth data were combined to understand the contribution of different barriers to RI. There are three main questions addressed in this study. (1) Do *E. secundum* and *E. xanthinum* hybridize in the wild, as indicated by the presence of specimens with intermediate morphological characters? (2) What is the strength of RI barriers between the parental species, and do hybrids bridge gene flow across the species boundaries? (3) What do the patterns of morphological diversity and genomic admixture tell us about speciation processes and species delimitation in the Neotropical *Epidendrum* genus? In addition, the role of pre- and postzygotic barriers in species cohesion and the potential evolutionary mechanisms associated with the maintenance of species barriers in *Epidendrum* are discussed.

MATERIALS AND METHODS

Plant material—The species investigated are found in the mountainous regions of South America. *Epidendrum secundum* has a very broad distribution, occurring in mountains within Planalto Brasileiro, Serra do Mar, Andean Cordillera, and Guiana Plateau (Hágsater and Soto-Arenas, 2005). Its extensive morphological, karyological, and genetic variation (Pinheiro and Barros, 2007; Pinheiro et al., 2009a, 2014) suggest the existence of highly differentiated lineages in this group. On the other hand, *E. xanthinum* is restricted to high elevations in the mountains of Southeastern Brazil, mainly in Serra dos Órgãos and Cadeia do Espinhaço. The phylogenetic study of Pinheiro et al. (2009a) used amplified length fragment polymorphism (AFLP) and plastid markers to show that *E. xanthinum* is a monophyletic lineage and a sister species to *E. secundum* specimens from different localities.

A total of 89 specimens of *E. secundum*, *E. xanthinum*, and putative hybrids were sampled in February 2011, at the Serra dos Órgãos mountains (Nova Friburgo, Rio de Janeiro state, Brazil), where distributions of *E. secundum* and *E. xanthinum* overlap. Here, despite the contrasting flower color (bright lilac flowers in *E. secundum* and yellow flowers in *E. xanthinum*), extensive morphological variations could be observed. Leaf fragments were collected and dried in silica gel. Total genomic DNA was extracted following the method described by Pinheiro et al. (2008a). To protect the orchid plants from illegal collectors, detailed geographic information of the sites sampled are not listed here, but are available from the authors upon request. Twenty-three specimens (seven *E. secundum*, nine *E. xanthinum*, and seven hybrids) were transferred to the orchid collection at the Instituto de Botanica, São Paulo, and used in the hand pollination experiments.

Microsatellite genotyping—All of the 89 collected specimens, including the individuals used in the manual crosses, were genotyped

FIGURE 1 Flower color polymorphisms found in *Epidendrum* plants sampled at the hybrid zone studied in the Nova Friburgo population. Plants were classified as *E. secundum* (A, B), hybrids (C, D, E, F) and *E. xanthinum* (G, H) based on the structure results (see text).

with eight nuclear microsatellite loci (Lspe03, Eff26, Eff45, Eff48, Eff49, Eff70, Epp56, and Epp96) isolated by Cortés-Palomec et al. (2008) and Pinheiro et al. (2008a, 2008b, 2013), and five plastid microsatellite loci (EPCP02, EPCP04, EPCP07, EPCP08, and EPCP09) isolated by Pinheiro et al. (2009b). Loci amplification and allele characterization were performed as described by Pinheiro et al. (2010).

Assignment analysis—Classification of *E. secundum* and *E. xanthinum* specimens, including the putative hybrid individuals, based on morphological characters was difficult because the phenotypic traits formed a continuous range of variation in the sympatric zone (Fig. 1). Thus, individuals were classified as parental species and hybrids using nuclear markers, as analyzed by Bayesian assignment tests. To estimate the nuclear admixture proportions of each individual, two Bayesian assignment methods were performed using structure v. 2.3.4 software (Hubisz et al., 2009) and TrueK (available at http://www.bobverity.com/truek/what-is-truek/). structure was used to assign each specimen to a genetic cluster (K) and to estimate the admixture proportions (q). The entire nuclear dataset was analyzed under the admixture model assuming correlated allele frequencies. The number of K was set from a minimum of one to a maximum of ten, and simulations were run for each K-value with a burn-in of 250,000 and 1,000,000 iterations each. The most probable number of genetic clusters (K) present in the data were analyzed using the methods proposed by Evanno et al. (2005) using the Structure Harvester v. 6.0 program (Earl and von Holdt, 2011), and the method implemented by the TrueK software. A threshold (Tq) of 0.9 was set to assign individuals as purebred of the parental species, $q \geq 0.9$ to *E. secundum* and $q \leq 0.1$ to *E. xanthinum*. Individuals with $0.9 > q > 0.1$ were assigned as hybrids.

Nuclear and plastid genetic diversity and structure—Nuclear microsatellite diversity parameters were characterized by calculating the number of alleles, allelic richness, expected and observed heterozygosity, and the inbreeding coefficient (Weir and Cockerham, 1984) per population. These parameters were calculated using GENEPOP 4.0 software (Raymond and Rousset, 1995). Each unique allelic combination found on the plastid microsatellite markers was attributed to a particular haplotype. The relationship between haplotypes was assessed using NETWORK v. 5.0 software (Bandelt et al., 1999) to construct a median-joining network. Under the model of maximum parsimony, one tree was recovered that contained the shortest, least complex, phylogenetic relationship among haplotypes. The diversity of the plastid DNA markers was assessed using the number of haplotypes detected in each species and in the hybrids, as well as the haplotype diversity and haplotype richness, estimated with RAREFAC v. 3.5 software (Petit et al., 1998). Estimates of haplotype richness were corrected for differences in sample size using the rarefaction method.

To explore the patterns and consequences of cytonuclear incompatibility in interspecific hybridization in *Epidendrum* species, departures from random cytonuclear associations, based on the structure assignment results and the plastid DNA haplotype data, were compared following Pinheiro et al. (2010), using the nonparametric Spearman rank correlations using SPSS 13.0 for Windows (SPSS).

Genome size estimation—A total of 12 individuals were used in this study: four *E. secundum*, four *E. xanthinum*, and four putative hybrids (according to the structure hybrid assignment test). These specimens were also used in the crossing experiments (see below). Sample nuclei were released by chopping 5 cm^2 of fresh leaf tissue together with 0.5 cm^2 of *Ruscus aculeatus* fresh leaf tissue (to act as an internal reference standard with 2C = 20.59 pg; Veselý et al., 2012) with a sharp razor blade in a Petri dish containing 1 mL WPB buffer. The nuclear suspension was recovered and filtered through a 50 µm nylon filter to remove cell fragments and large debris. The nuclei were stained with 50 mg mL^{-1} propidium iodide, and 50 mg mL^{-1} RNase (Sigma, St Louis, MO, USA) was added to the nuclear suspension to prevent staining of double-stranded RNA. Samples were stained for 40 min to 1 h prior to analysis. Flow cytometry was performed on a BD Biosciences FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA), and data were collecting using CellQuestPro software (Becton Dickinson, San Jose, CA, USA). Samples were run on low or medium pressure for long enough to acquire clear peaks (1–2 min). Because propidium iodide (emission maximum 639 nm) was used to stain the DNA, a FL2 detector (585 nm/42 nm) was used to measure fluorescence. The FL2-Area (FL2-A), a measure of the integrated fluorescence signal, was used as the parameter that correlated linearly to DNA content. Histogram data were collected and mean peak analysis was performed using Flowing Software 2.5.1 (www.flowingsoftware.com). Nuclear DNA content was calculated (sample peak mean divided by the standard peak mean) as the 2C nuclear DNA content of the standard in picograms (Doležel et al., 2007). For each sample, three replications were prepared independently, usually on different days, for flow cytometry analyses. As a quality control, only CV values (coefficient of variation for genome size estimation) of G0/G1 peaks < 5% the analyses were retained (Vega et al., 2013); otherwise the sample preparation was repeated.

Crossing experiments—Manual crossing experiments were performed on specimens of *E. secundum*, *E. xanthinum*, and the putative hybrids, under controlled conditions to avoid pollinator interference. The classification of all specimens used in the crossing experiments was based on the nuclear assignment results (see above). Hand pollination was performed by removing all four of the pollinia from one donor flower with a metallic pin and placing them on a single stigma of the recipient plant that was either of the same species (intraspecific cross—used as a control), of the sympatric heterospecific species (interspecific cross), or of the hybrid (backcross). Self-pollination using one hybrid plant was also performed. All of the possible crosses between *E. secundum*, *E. xanthinum*, and hybrids were performed, for a total of 10 cross types. In each intraspecific and interspecific experiment, 3–13 plants were used. Three flowers per specimen were used on each cross, and crosses were performed bidirectionally, with each flower providing and receiving pollen from the intraspecific or interspecific partner, respectively. In total, 202 flowers from 23 plants were crossed.

Seed viability measures—Fruit development was monitored until the fruits were mature (as evidenced by opening of ripe fruits). Fruit set was measured by dividing the number of mature fruits by the total number of pollinated flowers. Then, mature fruits were collected and checked for the presence of seeds, which were immersed in a 1% solution of 2,3,5-trifenil tetrazolium and stored for 24 h at 30° C. Following this procedure, viable embryos were stained a strong red color. At least 300 seeds from each fruit were analyzed under the microscope in three independent counts. The percentage of viable seeds was calculated by dividing the number of viable embryos by the total number of embryos scored. Fruit and

seed data were obtained for each cross type and compared using Mann–Whitney or Kruskal–Wallis tests with SPSS 11.0 software.

The strength of three postmating barriers were estimated following Scopece et al. (2013), and modified according to Sobel and Chen (2014): pollen–stigma compatibility (prezygotic), seed viability (early postzygotic), and hybrid viability (late postzygotic). Fruit set results were used to estimate the strength of pollen–stigma compatibility, and embryo viability was estimated using seed viability measures. The strength of pollen–stigma compatibility was defined as $RI_{\text{pollen-stigma}} = 1 - 2^*$ (percentage of fruit formed in interspecific crosses/percentage of fruit formed in intraspecific + interspecific crosses). The strength of embryo viability was defined as $RI_{\text{embryo viability}} = 1 - 2^*$ (percentage of viable seeds in interspecific crosses/percentage of viable seeds in intraspecific + interspecific crosses). 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.

Fruit set and seed viability obtained from backcrosses (parental species × hybrids) were considered for two stages of hybrid viability. Thus, the strength of hybrid viability was calculated separately for each stage. For each species, the strength of hybrid viability based on the fruit set stage was defined as $RI_{\text{hybrid\_F}} = 1 - 2^*$(percentage of fruit formed in backcross/percentage of fruit formed in backcross + the maximum hypothetical fruit set). Similarly, the strength of hybrid viability based on the seed viability stage was defined as $RI_{\text{hybrid\_S}} = 1 - 2^*$(percentage of seed viability in the backcross/percentage of seed viability in the backcross + the maximum hypothetical seed viability). In both cases, the maximum hypothetical fruit set and seed viability values were equal to 1. Following Scopece et al. (2013), $RI_{\text{hybrid\_F}}$ and $RI_{\text{hybrid\_S}}$ were combined for all the crosses as $RI_{\text{hybrid\_FS}} = RI_{\text{hybrid\_F}} + (1 - RI_{\text{hybrid\_F}})^* RI_{\text{hybrid\_S}}$. The strength of male and female hybrid viability was calculated independently for crosses in which the hybrids acted as pollen donors or seed parents, respectively. The mean of the male and female indices was used to calculate the total strength of hybrid viability ($RI_{\text{hybrid sterility}}$).

The three indices ($RI_{\text{pollen-stigma}}$, $RI_{\text{embryo viability}}$, and $RI_{\text{hybrid sterility}}$) were merged following the methods proposed by Sobel and Chen (2014), using the equation RI_{4E}. We calculated the relative strength and absolute contribution of each barrier for one direction of gene flow, assuming that interspecific gene flow may be asymmetric (Sobel and Chen (2014). All measures of isolation varied between 0 (no isolation) and 1 (complete isolation). The strength of RI of a particular stage was considered asymmetric when the difference between the two possible directions was higher than 0.25 (Scopece et al., 2013).

Pollen tube growth experiment—To investigate whether or not pollen tubes would reach the ovary in different types of crosses, we performed a second set of hand pollinations involving both parental species and hybrids. Two individuals of each parental species were used to perform intraspecific and interspecific crosses, including backcrosses using two hybrids. Flowers were fixed in 50% FAA at 10–12 d after pollination (Moraes et al., 2013) for later observation of pollen grain germination and for evaluation of the morphology and growth of the pollen tubes using epifluorescence microscopy (Martin, 1959). 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).

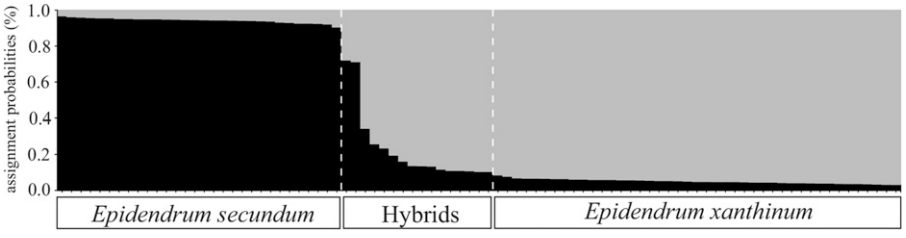

FIGURE 2 Genetic assignment results obtained using structure, including posterior probabilities (*q*) for *E. secundum*, *E. xanthinum*, and hybrids. Each vertical bar represents an individual. The proportion of color in each bar represents an individual's assignment probability corresponding to *E. secundum* (black) and *E. xanthinum* (gray).

RESULTS

Genetic structure and hybrid detection—The number of genetic groups in our sample was confirmed as $K = 2$ using the method of Evanno et al. (2005) and TrueK software (Appendix S1A and S1B, respectively, see Supplemental Data with the online version of this article). Using a value of $K = 2$, the Bayesian analyses performed by structure consistently identified purebred individuals corresponding to *E. secundum* and *E. xanthinum*, and specimens showing admixed ancestry were identified as hybrids. The admixture proportions, *q*, for each individual are shown in Fig. 2. Considering a *Tq* value of 0.9, 16 hybrids and 73 purebred specimens (30 *E. secundum* and 43 *E. xanthinum*) were identified.

Genetic diversity of parental and hybrid plants—Purebreds and hybrid individuals sampled at the same region were assigned according to the structure admixture results. The diversity parameters were high for both parental species and hybrids (Table 1), whereas the diversity was slightly lower in *E. xanthinum* than in the hybrids and *E. secundum*. All eight loci were polymorphic, except for one monomorphic locus in *E. xanthinum* (Eff48). The number of alleles varied between two and nine in *E. secundum* and the hybrids, and between one and six in *E. xanthinum*. The allelic richness and private allele richness of the hybrids displayed intermediate values between those of the purebred populations. The expected and observed heterozygosity ranged from 0.042–0.851 and from 0.042–0.875, respectively. Three loci displayed significant departures from the Hardy–Weinberg Equilibrium (HWE) because of heterozygote deficits in the purebred species and in five of the hybrids. Low, but significant, values of inbreeding coefficient were observed for the parental species and hybrids (Table 1).

Plastid genetic structure—A total of 21 plastid haplotypes were found (Appendix S2). Two were removed from the analysis because they were only identified in one individual (singletons). The haplotype network showed two distinct clusters of haplotypes, one belonging to *E. secundum* and one to *E. xanthinum* (Fig. 3, Table 2). Each cluster was separated from the other by at least six mutational steps, whereas haplotypes belonging to the same cluster were separated from each other by one mutational step in most cases. In general, most of the plants included in the *E. secundum* clade also showed nuclear admixture proportions of the pure *E. secundum*, and a similar pattern was observed for

TABLE 1. Genetic diversity results obtained for *Epidendrum secundum*, *E. xanthinum*, and hybrids, using eight nuclear and five plastid microsatellite markers, including the number of individuals sampled (*N*), number of alleles (A), allelic richness (AR), the expected (H_E) and observed (H_O) heterozygosities, the within population inbreeding coefficient *f* for nuclear microsatellites, the number of plastid DNA haplotypes found (NH), the haplotype diversity (HD) and haplotype richness (HR).

	Nuclear microsatellites					Plastid microsatellites		
Species (*N*)	A	AR	H_E	H_O	*f*	NH	HD	HR
E. secundum (30)	43	4.795	0.619	0.539	0.119*	10	0.887	7.186
Hybrids (16)	35	4.279	0.620	0.497	0.184*	9	0.858	8.000
E. xanthinum (43)	30	3.319	0.528	0.375	0.215*	11	0.857	6.889

*Departures of within-population inbreeding coefficients (*f*) from the Hardy–Weinberg equilibrium (HWE) are indicated by asterisks ($P < 0.0001$).

plants grouped in the other clade, which was mostly composed of pure *E. xanthinum*. Hybrids were observed in both clades. The H19 haplotype was present in the *E. secundum* clade, and all of the plants carrying this haplotype showed nuclear admixture proportions of *E. xanthinum* and the hybrids. Most hybrids (14) contained *E. xanthinum*-specific haplotypes, and only two hybrids contained *E. secundum* haplotypes (Table 2). A significant correlation was detected between the nuclear and plastid genomic composition (Spearman's $r = 0.571$, df = 87, $P < 0.001$), suggesting cytonuclear incompatibilities.

Flow cytometry data—The mean genome size estimated in pure parental species was 3.77 ± 0.19 pg/2C in *E. secundum* and 4.27 ± 0.15 pg/2C in *E. xanthinum*. The hybrids' genome size ranged from 3.00–3.95 pg/2C, suggesting that the parental contribution to the *hybrid genome* was unequal. The genome size only showed a significant difference between *E. xanthinum* and the hybrid plants (ANOVA test, $F = 11.314$, df = 2, 9, $P < 0.01$ followed by a Tukey test $P < 0.01$).

Strength of RI barriers—The majority of intraspecific pollinations resulted in mature fruits, as indicated by the mean fruit set values: 0.68 ± 0.47 standard deviation (SD) in *E. secundum*, and 0.88 ± 0.32 SD in *E. xanthinum*. The mean fruit set of interspecific crosses when *E. xanthinum* was the pollen recipient did not differ from *E. xanthinum* intraspecific crosses (U = 279.000, df = 49, $P = 0.198$). Backcrosses to *E. xanthinum* (pollen recipient) also did not differ in mean fruit set from intraspecific crosses (U = 214.500, df = 42, $P = 0.543$). Mean fruit formation pooled for *E. secundum* and *E. xanthinum* intraspecific crosses ($N = 59$, 0.77 ± 0.41 SD) was 25% significantly higher (U = 1138.000, df = 105, $P = 0.029$) when compared to the pooled fruit formation data for the interspecific crosses ($N = 48$, 0.58 ± 0.49 SD). Similarly, the mean fruit formation pooled for *E. secundum* and *E. xanthinum* intraspecific crosses was significantly higher—32% (U = 1759.000, df = 137, $P = 0.002$)—when compared to crosses in which the hybrids received or donated pollen to the parental species ($N = 80$, 0.52 ±0.50 SD). Seed viability from

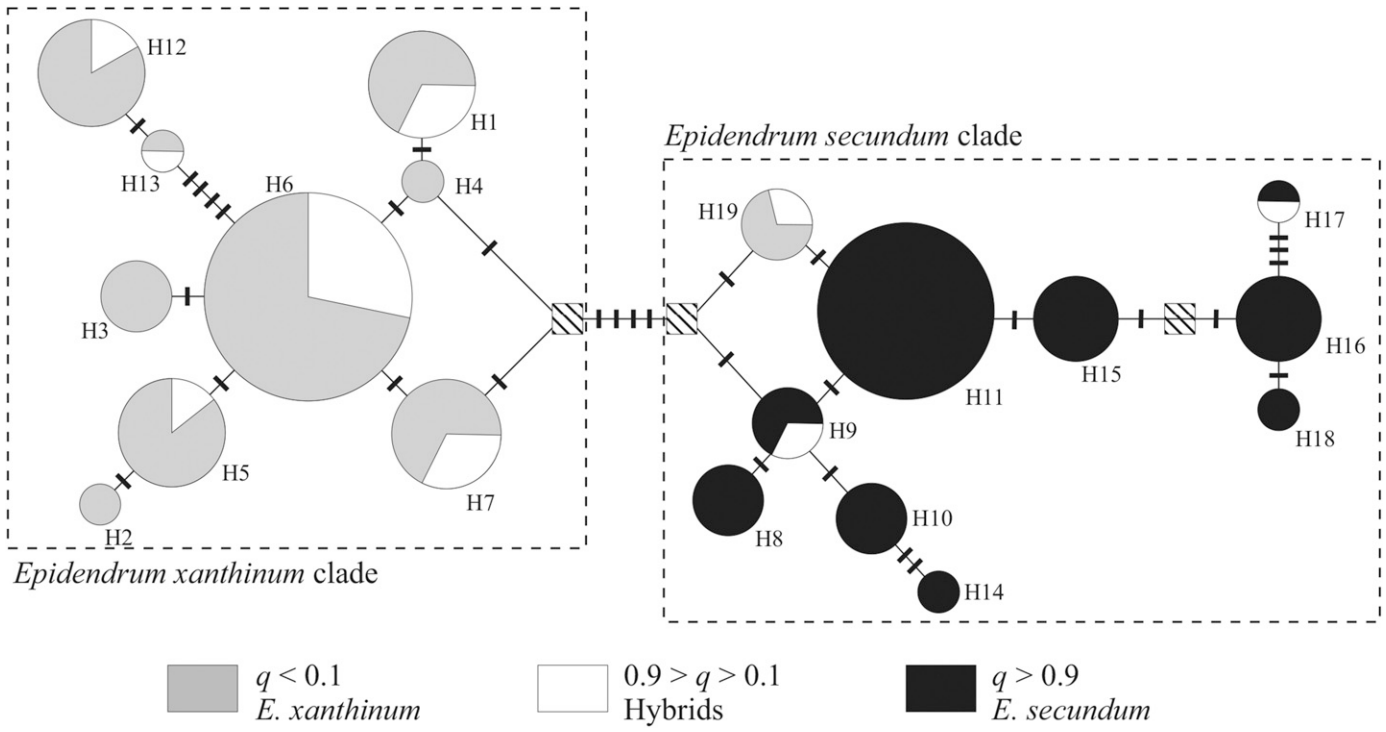

FIGURE 3 Median-joining network of plastid DNA haplotypes found in *E. secundum* (black), *E. xanthinum* (gray), and hybrids (white). The haplotypes are indicated by filled circles, and the size of each circle is proportional to the observed frequency of each type. The proportion of colors in each haplotype indicates the amount of individuals classified according to the structure assignment probabilities (*q*). The number of mutations required to explain the transitions among haplotypes is indicated by cross hatches along the lines connecting the haplotypes. Striped squares indicate missing intermediate haplotypes not found in the individuals analyzed.

TABLE 2. Plastid DNA haplotypes (H) found in 89 individuals from the *Epidendrum secundum* / *E. xanthinum* hybrid zone, including frequencies in both species and hybrids considering the structure assignment probabilities and range of nuclear admixture proportions for plants carrying each haplotype.

		Frequencies (structure assignment results)			
H	Haplotype group	*E. secundum* (*N* = 30)	Hybrids (*N* = 16)	*E. xanthinum* (*N* = 43)	Structure admixture range
H8	*E. secundum*	3	0	0	0.938–0.952
H9	*E. secundum*	2	1	0	0.712–0.948
H10	*E. secundum*	3	0	0	0.926–0.967
H11	*E. secundum*	8	0	0	0.925–0.955
H14	*E. secundum*	2	0	0	0.930–0.944
H15	*E. secundum*	4	0	0	0.905–0.950
H16	*E. secundum*	4	0	0	0.945–0.962
H17	*E. secundum*	1	1	0	0.721–0.950
H18	*E. secundum*	2	0	0	0.939–0.944
H21	*E. secundum*	1	0	0	0.957
H1	*E. xanthinum*	0	2	4	0.034–0.136
H2	*E. xanthinum*	0	0	2	0.037–0.045
H3	*E. xanthinum*	0	0	3	0.043–0.067
H4	*E. xanthinum*	0	0	2	0.045–0.048
H5	*E. xanthinum*	0	1	5	0.042–0.195
H6	*E. xanthinum*	0	6	14	0.032–0.344
H7	*E. xanthinum*	0	2	4	0.040–0.137
H12	*E. xanthinum*	0	1	5	0.048–0.162
H13	*E. xanthinum*	0	1	1	0.042–0.110
H19	*E. xanthinum*	0	1	2	0.041–0.235
H20	*E. xanthinum*	0	0	1	0.051

For the structure assignment probabilities, we considered pure *E. secundum*, pure *E. xanthinum*, and hybrids plants with posterior probabilities of $q \geq 0.90$, $0.10 \leq q \leq 0.90$, and $q \leq 0.10$, respectively.

intraspecific crosses was variable within each species, and ranged from 16.0–99.1% in *E. secundum* and from 42.3–97.0% in *E. xanthinum* (Table 3). Mean seed viability was higher, but not significantly so (U = 596.000, df = 72, $P = 0.592$) in the intraspecific crosses (0.74 ± 0.21 SD) when compared to the interspecific crosses (0.59 ± 0.39 SD). Experiments in which hybrids were crossed to parental species (both with hybrids acting as pollen donors and recipients) resulted in significantly lower seed viability than intraspecific crosses (U = 239.000, df = 91, $P < 0.001$). Mean seed viability was higher when hybrids acted as pollen donors to the parental species, as compared to values observed when hybrids acted as pollen recipients (Table 3), although this result was not significant when considering comparisons between hybrids and *E. secundum* (U = 36.000, df = 20, $P = 0.152$) and between hybrids and *E. xanthinum* (U = 24.000, df = 18, $P = 0.137$). Self-pollinations performed with hybrids and crosses between different hybrid plants showed an average fruit formation of 41.6%, but with low levels of seed viability, ranging from 0–5.6%.

The RI indices presented here represent parental species and hybrids acting as seed parents. There was strong but incomplete RI between *E. secundum* and *E. xanthinum*, with a combined postmating isolation index value of 0.866 (Table 4). The total RI values calculated for each parental species were similar; 0.919 for *E. secundum* and 0.813 for *E. xanthinum*. However, individual barriers showed different contributions for each species. Hybrid sterility was the strongest barrier in both species, with values of 0.521 for *E. secundum* and 0.657 for *E. xanthinum*. In *E. secundum*, almost half the absolute contribution of RI was due to hybrid sterility, which was evident from the low levels of seed viability observed in the backcrosses with the hybrids (Table 3). On the other hand, the absolute contribution of embryo viability was very low for *E. secundum* (0.049). Pollen–stigma compatibility was the barrier that contributed the least to overall RI in *E. xanthinum* (0.108). This was the only asymmetric barrier identified between *E. secundum* (0.424) and *E. xanthinum* (0.132), showing a difference in relative strength higher than 0.25 (Table 4).

Pollen germination and pollen tube growth was observed in flowers from all of the crossing experiments (Appendix S3A–S3D), except in those where the fruits were aborted. In general, pollen grains germinated in large quantities, and their pollen tubes grew straight and had callose plug deposits regularly spaced along their lengths. The pollen tubes reached the base of the column and ovary in all of the nonaborted fruits analyzed (Appendix S3D).

TABLE 3. Fruit formation and viable seeds produced from hand pollination of *Epidendrum secundum*, *E. xanthinum*, and hybrids, including the number of plants used as seed parents and pollen donors (N), the number of pollinated flowers (Flower), number of fruits produced (Fruit), the ratio between fruits produced by pollinated flowers (FR/FL), and seed viability (SV).

Pollen receptor (N)	Pollen donor (N)	N[1]	Flower	Fruit	FR/FL (SD)[1]	SV (SD)[2]
Intraspecific crosses						
E. secundum (7)	*E. secundum* (7)	7	32	22	0.68 (0.47)a,d,e	0.65 (0.25)a
E. xanthinum (6)	*E. xanthinum* (6)	6	27	24	0.88 (0.32)a	0.82 (0.13)b
Interspecific crosses						
E. secundum (7)	*E. xanthinum* (6)	13	24	10	0.41 (0.50)b,c	0.57 (0.41)a,b,c,g
E. xanthinum (6)	*E. secundum* (7)	13	24	18	0.75 (0.44)a,e	0.59 (0.40)a,b,d
Backcrosses with *E. secundum*						
E. secundum (7)	Hybrid (4)	11	24	8	0.33 (0.48)c	0.37 (0.40)a,e,f
Hybrid (4)	*E. secundum* (7)	11	24	14	0.58 (0.50)c,d,e,f	0.08 (0.10)e
Backcrosses with *E. xanthinum*						
E. xanthinum (4)	Hybrid (4)	8	17	14	0.82 (0.39)a,f	0.39 (0.38)c,d,f
Hybrid (4)	*E. xanthinum* (4)	8	15	6	0.40 (0.50)c,d	0.09 (0.10)c,e,f,g
Crosses using hybrids only						
Hybrid (3)	Hybrid (3)	3	12	5	0.41 (0.51)c,d,e	0.02 (0.02)e,g
Hybrid self-pollination (1)*		1	3	1	0.33 (0.00)	0.00

[1]Total number of plants used; [2]The same letter indicates that the means are not significantly different ($P > 0.05$); *Not used for statistical tests.

TABLE 4. Estimates of the relative strength (Str) and absolute contribution (AC) of RI between *Epidendrum secundum* and *E. xanthinum*, considering one prezygotic barrier (pollen-stigma compatibility) and two postzygotic barriers (embryo viability and hybrid viability). All measures of isolation varied between 0 (no isolation) and 1 (complete isolation).

	Prezygotic		Postzygotic				
	$RI_{pollen\text{-}stigma}$		$RI_{embryo\ viability}$		$RI_{hybrid\ viability}$		
Species	Str	AC	Str	AC	Str	AC	Total RI
E. secundum[1]	0.424	0.390	0.054	0.049	0.521	0.480	0.919
E. xanthinum[1]	0.132	0.108	0.209	0.170	0.657	0.534	0.813
RI stage	0.278	0.249	0.131	0.109	0.589	0.507	0.866
Asymmetry[2]	0.292		0.155		0.136		

[1]RI values calculated when species act as pollen recipients; [2]The asymmetry was calculated based on the difference of the relative strength of RI between *E. secundum* and *E. xanthinum*, for each barrier, following Scopece et al. (2013).

DISCUSSION

Hybridization between *E. secundum* and *E. xanthinum* was confirmed, supporting previous hybrid reports in the subgenus *Amphyglottium* (Dunsterville, 1979; Dressler, 1989; Vega et al., 2013). Nuclear markers indicated different levels of admixture between the parental species and hybrids, and the plastid data set revealed that both parental species acted as seed parents in the hybridization events. Fruits and viable seeds were formed in interspecific crosses and in crosses including hybrids as pollen donors and recipients, confirming the existence of permeable barriers between parental species. Indeed, the hybrid plants showed extensive variation in flower color patterns, which were intermediate between *E. secundum* (pink flowers) and *E. xanthinum* (yellow flowers). Nuclear DNA content appeared to be influenced by hybridization events, because the genome size values were variable within the parental species and hybrids. As observed in previous studies, differences in ploidy levels do not preclude hybridization and introgression events in *Epidendrum*, particularly in species of the subgenus *Amphyglottium* (Pinheiro et al., 2010; Vega et al., 2013, Moraes et al., 2013). Despite the permeability of the pre- and postzygotic barriers, haplotype sharing between parental species was not observed in this study, indicating that cytonuclear incompatibilities might prevent interspecific gene flow. Cytonuclear incompatibilities were observed in other *Epidendrum* hybrid zones (Pinheiro et al., 2010; Vega et al., 2013) and may constitute an important component of RI between *E. secundum* and *E. xanthinum*.

Species integrity and cohesion—Molecular markers are crucial to defining pure specimens of *E. secundum* and *E. xanthinum*. A wide diversity in flower characters was observed in the hybrid zone investigated in this study (Fig. 1), and identification of the plants in the field was virtually impossible. Hybrids normally show a broad range of flower colors, leading to uncertainties in the morphological identification of the parental species. Pinheiro et al. (2010) observed a similar pattern in the *E. fulgens* and *E. puniceoluteum* hybrid zones, where some plants identified in the field as parental species were recognized as hybrids through the use of molecular markers. In fact, species delimitation within the subgenus *Amphyglottium* clade proved challenging in the past (Dunsterville and Garay, 1961; Hágsater, 1984; Dressler, 1989). Most of the species described in this group were delimited based on details of flower morphology and color. The taxonomic history of this group contains many different opinions regarding species delimitation. Some authors, such as Dunsterville and Garay (1961), considered *E. secundum* and *E. xanthinum* to be synonymous, because of the extensive variation observed in the natural populations, where floral morphological discontinuities are difficult to ascribe. Others, such as Pabst and Dungs (1975) and Dressler (1989), considered flower color to be an effective characteristic for distinguishing between both species, despite the presence of intermediates.

Population level studies have shown extensive variation in floral characters within *Epidendrum* populations (Pinheiro and Barros, 2007). At the same time, hybridization events in this group have been reported in the literature, based on the large morphological variation observed in some populations, which were considered hybrid swarms between two or more species (Dunsterville, 1979; Dressler, 1989). Specific tests that explored the hypothesis of hybridization in this group were provided by Vega et al. (2013), and revealed extensive hybridization among the Andean species of *E. calanthum*, *E. cochlidium,* and *E. schistochilum*. Vega et al. (2013) also confirmed that most of the morphological variation observed in the sampled populations was explained by hybridization.

In this case study, we conducted assignment tests based on nuclear microsatellites that indicated two clear genetic groups (Appendix S1A–S1B), mainly composed of pure *E. secundum* and *E. xanthinum* plants (Fig. 2). It is important to note that most plants genetically identified as pure *E. secundum* had lilac flowers, and the same pattern was observed in *E. xanthinum*, where pure individuals had yellow flowers. However, hybrid plants had flowers with different color patterns, including lilac, yellow, and orange tones. Taken together with the results of other population-level studies in *Epidendrum* (Pinheiro et al., 2010; Vega et al., 2013; Marques et al., 2014), the data in this study add to the growing body of evidence suggesting that hybridization plays an important role in the morphological diversity in this group, blurring boundaries among closely related species. Moreover, the contrasting flower color of parental species may suggest the existence of selection mediated by different pollinators—a potential premating barrier that should be investigated in the future.

Despite the gradient of variation observed in the flower characters (Fig. 1) and genome size, two distinct genetic groups were observed. Several plants were identified as pure parental species, suggesting that barriers to gene exchange are present to some extent. Premating barriers were not tested here, but they are potentially permeable, as shown by the number of hybrid plants found and the evidence of introgression. The same pattern is commonly observed in food-deceptive orchids, where different orchids share a large array of pollinator species (Cozzolino and Scopece, 2008). Scopece et al. (2010) observed a higher efficiency in intraspecific pollination events among rewarding species compared to food deceptive species. It is possible that low pollinator sharing is likely the basis for efficient pollination rates in particular groups of rewarding species and sexually deceptive orchids (Scopece et al., 2010). Many *Epidendrum* species are described as food-deceptive and as being primarily pollinated by Lepidoptera (reviewed by Pinheiro and Cozzolino, 2013); some studies have reported more than 20 different butterfly species acting as pollinators of *E. secundum* (Pansarin and Amaral, 2008). The pollinators of *E. xanthinum* are unknown, and pollination mechanisms in this species need to be addressed in future studies. Of particular interest will be measures of visitation rates and pollen removal among parental species and hybrids (Ren et al., 2014), which could clarify the role of premating

barriers and the role played by selection pressure on floral characters (Jacquemyn et al., 2012), including differences in flower color (Ramsey et al., 2003; Palma-Silva et al., 2015).

Reproductive barriers to gene exchange between parental species—Postmating barriers were almost complete between *E. secundum* and *E. xanthinum* (Table 4). The level of hybrid sterility was higher in both parental species, indicating the prevalence of late postzygotic barriers compared to earlier stages (Table 4). This result was expected given the genomic differences observed between *E. secundum* and *E. xanthinum*. Both parental species showed considerable differences in ploidy levels within the sympatric population studied here (*E. secundum* $2n = 4\times = 56$, *E. xanthinum* $2n = 2\times = 28$, Assis, 2013) and in the genome size (*E. secundum* = 3.77 pg/2C and *E. xanthinum* = 4.27 pg/2C), suggesting that some hybrids might suffer from severe meiotic problems. Despite the possible meiotic issues associated with different ploidy levels, some hybrids between *E. secundum* and *E. xanthinum* were fertile, producing fruits and viable seeds (Table 3), as observed in other *Epidendrum* hybrid zones (Moraes et al., 2013; Pinheiro et al., 2015). Moreover, the slight differences in genome size of the two parental species excludes the possibility of differences at the ploidy level.

Hybrids may bridge interspecific gene exchange in different ways, depending on which parental species is involved in the backcross. Total RI was higher in *E. secundum* (Table 4), and introgression is likely to be less common in this species. In fact, fruit abortion was higher when *E. secundum* received pollen from *E. xanthinum*, substantially decreasing the amount of seeds produced and, consequently, the number of hybrids carrying *E. secundum* haplotypes (Fig. 3, Table 2). Hybrid sterility was lower in *E. secundum*, but it apparently did not affect the patterns observed in assignment tests, which indicate a higher influence of the *E. xanthinum* genome in the admixture patterns observed in the hybrids (Fig. 2). Thus, the higher pollen–stigma compatibility observed in *E. xanthinum* may offer more opportunities for the generation of F1 hybrids produced by different parental plants, increasing the number of hybrids carrying *E. xanthinum* haplotypes (Fig. 3, Table 2) and the occurrence of introgression toward *E. xanthinum* (Fig. 2). Examples in the literature show patterns of asymmetric introgression toward the parental species showing the higher chromosome number (Ståhlberg and Hedrén, 2009; Pinheiro et al., 2010). However, this was not the case here because *E. xanthinum* had the smaller number of chromosomes and genome size (see above). This potential asymmetric introgression pattern could be influenced by the different frequencies of the parental species in the sympatric region. This hypothesis is supported by empirical studies showing that more numerous species will be protected from introgression by the higher levels of intraspecific gene exchange (Petit and Excoffier, 2009). In this scenario, the rarer species will behave more often as a pollen recipient in the F1 formation and introgression. Indeed, *E. secundum* is the more abundant species, and has a longer flowering period (almost a whole year). On the other hand, populations of *E. xanthinum* are smaller and less abundant, which probably influenced the lower genetic variation and higher inbreeding coefficient observed for this species (Table 1). In addition, *E. xanthinum* blooms during the rainy season, from December to March, indicating a reduced window for intraspecific gene exchange to limit introgression.

***Genetic mechanisms underlining the RI between* E. secundum *and* E. xanthinum**—The fixation of incompatible loci, as predicted by the BDM classic model of genic incompatibilities (Coyne and Orr, 2004), also plays an important role in reducing interspecific recombination and gene exchange in this hybrid zone. Interestingly, genic incompatibilities usually interfere with male fertility, such as anther development and pollen viability (reviewed in Schnable and Wise, 1998). To date, male infertility is also the major source of sterility in *Epidendrum* hybrids (Pinheiro et al., 2010, 2015; Vega et al., 2013; Marques et al., 2014). However, the results observed in *E. secundum* × *E. xanthinum* hybrids contradicted this expectation. Normal pollen tube growth was observed when hybrids acted as pollen donors in crossing experiments (Appendix S3). Moreover, seed viability was lower when parental species acted as pollen donors to the hybrid plants (Table 3), suggesting a genetic mechanism that decreases the viability of hybrid ovules. Interspecific crosses between rice species revealed an important isolation barrier in which three linked loci interact, also known as the "killer/protector package" (Ouyang and Zhang, 2013). In this mechanism, the hybrid sterility and preferential abortion of female gametes are controlled by the complex interaction between "killer" genes (ORF5 and ORF4) and a "protector" gene (ORF3). This mechanism has rarely been reported in nonmodel organisms (Baack et al., 2015), and its occurrence in *Epidendrum* should be investigated by future studies.

When parental species show large chromosome differences, the origin of hybrid sterility can be connected to chromosomal rearrangements or genes contained within the rearrangements (Navarro and Barton, 2003). Inducing chromosome doubling in *Epidendrum* hybrids could provide a simple test of whether decreased F1 fertility is due to chromosomal or genic factors. If chromosome doubling restores hybrid fertility, the most probable origin of F1 decreased fertility would be due to problems in meiotic pairing rather than genes within the rearranged segments of chromosomes, as observed in *Mimulus* hybrids (Stathos and Fishman, 2014). No hybrids with duplicated genomes were detected in this study, and this has also not been reported for other *Epidendrum* hybrid zones (Vega et al., 2013; Moraes et al., 2013; Marques et al., 2014; Pinheiro et al., 2015). Studies using a larger sample size, such as those conducted by Trávníček et al. (2011), would clarify the occurrence of genomic duplication in *Epidendrum* hybrid zones.

Negative cytonuclear interactions may constitute another source of genic incompatibility that decreases the fertility of *Epidendrum* hybrids. Cytonuclear incompatibility was significant between *E. secundum* and *E. xanthinum*, and this pattern was evident in the lack of haplotype sharing between species (Fig. 3, Table 2). Pure *E. secundum* individuals, identified using nuclear markers, do not carry haplotypes from *E. xanthinum* and vice versa, suggesting the presence of barriers to prevent the interaction of nuclear and plastid genes in late generation hybrids. Both parental species showed extensive differences in the nuclear and organellar alleles, a potential product of long, independent evolutionary trajectories after lineage divergence. The negative effects of nuclear–organellar interactions are generally considered to be a primary cause of intrinsic postmating reproductive barriers in plants (Martin and Willis, 2010; Greiner et al., 2011; Palma-Silva et al., 2011), and the mechanisms involved in the origin of these incompatibilities are of particular interest.

Selection for different environments and pollinators are evolutionary factors that could be involved in the differentiation observed between the flowering species (Abbott et al., 2013). According to Baack et al. (2015), most reproductive barriers are potential products of natural selection, but this remains to be explored in nonmodel plants, such as *Epidendrum*. However, genetic drift cannot

be ruled out as an important mechanism contributing to the genetic differentiation of both species, particularly when the fragmented nature of populations and the small effective population size of plastid markers are considered (Ellstrand, 2014). Species inhabiting highly fragmented landscapes, such as the mountains where *E. secundum* and *E. xanthinum* are found, are expected to accumulate higher levels of genetic differentiation, as illustrated elsewhere (Palma-Silva et al., 2011; Pinheiro et al., 2014).

CONCLUSIONS

Extensive genetic differences and reproductive barriers were found between *E. secundum* and *E. xanthinum*, clarifying the species limits in this sympatric zone. Indeed, most of the taxonomic uncertainties were concentrated in sympatric populations where co-occurring plants exhibited intermediate floral characters, suggesting the occurrence of hybridization. Barriers to gene exchange were most evident at the postzygotic stage, such as seed and hybrid viability, preventing species collapse. The lack of haplotype sharing and significant cytonuclear incompatibility may indicate negative interactions between the nuclear and plastid genes in hybrid plants, decreasing the fertility observed in these plants. Despite the strength of the reproductive barriers, fertile seeds were produced in some backcrossing experiments, and the existence of interspecific gene exchange cannot be excluded. Indeed, interspecific gene exchange may play an important role in isolated plant populations, where intraspecific gene exchange is limited, as shown in other studies (Palma-Silva et al., 2011, 2015). Despite the extensive morphological variability found, genetically pure parental species showed contrasting flower colors, and the occurrence of premating barriers associated with different pollinator preferences should be investigated in the future. Our results suggest that the morphological and molecular variability observed in hybrid plants are an important evolutionary outcome, rather than a simple taxonomic bias. Progress toward understanding the importance of different barriers and their evolution can be made by focusing on the fitness of hybrid plants and parental species under natural conditions. Whether selection pressure from different pollinators or habitats, and/or genetic drift are important mechanisms involved in the origin of RI between *E. secundum* and *E. xanthinum* is an important question which will offer interesting *avenues for future research*.

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