Evolutionary Ecology
https://doi.org/10.1007/s10682-020-10095-5

ORIGINAL PAPER

Reproductive barriers and fertility of two neotropical orchid species and their natural hybrid

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Received: 18 August 2020 / Accepted: 21 December 2020

Abstract

Different pre- and postzygotic isolating mechanisms may prevent interspecific gene exchange in secondary contact zones. Due to the different nature of each isolating barrier, which may act in different life history stages, multidisciplinary approaches are crucial to investigate the evolution of reproductive isolation (RI) in contact zones. In this study, we analysed seven different pre- and postzygotic RI mechanisms and reproductive success of two neotropical orchid species with contrasting pollination strategies, the nectarless food-deceptive *Epidendrum denticulatum* and the nectar rewarding *E. orchidiflorum*. The two species occur sympatrically in the coastal vegetation of Southeastern Brazil and share habitats with their natural hybrid *E.* x *purpureum*. Our aim was to test the contribution of pre and postzygotic reproductive barriers to species cohesion, examining potential asymmetries among RI mechanisms. Our results indicate habitat isolation as an important prezygotic barrier, strongly influenced by the contrasting habitat preferences found between the parental species. Hybrid sterility was also important, though incomplete, to prevent species collapse in this hybrid zone. This latter barrier was likely shaped by strong differences in chromosome numbers found between parental species (*E. denticulatum* 2n = 52, *E. orchidiflorum* 2n = 156). Indeed, hybrids showed lower levels of fertility when compared to parental species, probably due to meiotic abnormalities found in hybrid plants. However, contrary to our expectations, hybrid plants are still able to attract flower visitors during the day and night, and natural pollination success was comparable to one of the parental species, suggesting sexual reproduction of hybrid plants may contribute to the persistence of this hybrid zone. This study highlights the importance of studying hybrid zones between species diverging in several morphological and ecological traits, where the balance of hybridization is still unpredictable and almost unknown.

Keywords Chromosome barriers · Fruit set · Hybridization · Inbreeding · Isolation mechanisms · Seed fertility

Supplementary Information The online version contains supplementary material available at (https://doi.org/10.1007/s10682-020-10095-5).

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Published online: 04 January 2021

Introduction

Climatic oscillations are responsible for the rearrangement of the geographic distribution of species (Hewitt 2000; Vallejo-Marín and Hiscock 2016). Such changes in species ranges may delimit periods of relative isolation that are followed by secondary contact. During geographic isolation, genetic drift, mutation, and divergent ecological selection may act alone or in concert to differentiate populations (Coyne and Orr 2004). These processes can shape genetic, phenotypic and ecological traits, hence creating discontinuity among diverging lineages. When these related lineages come back into secondary contact, the eventual restoration of gene flow may generate progeny of mixed ancestry in hybrid zones (Barton and Hewitt 1985). In these hybrid zones, different pre- and/or postzygotic reproductive barriers may act alone or in concert preventing the disappearance of species boundaries (Grant 1981). However, these barriers may be also permeable allowing for substantial interspecific gene exchange when fertile hybrids co-occur with parental species (Levin 2012).

The extent to which reproductive barriers are permeable is extremely variable and determine the fate of the hybrid zone (Lowry et al. 2008). When RI is weak, high levels of introgression and even parental species extinction can occur (Todesco et al. 2016; Zitari et al. 2012), whilst when RI is strong, high levels of sterility are generally found in hybrid plants, precluding or limiting gene exchange between parental species (e.g., Scopece et al. 2013; Pinheiro et al. 2015; Shang et al. 2020). In these latter hybrid zones, i.e. those between well separated species with high RI, the emerging picture arising from the literature is that a combination of pre and postzygotic barriers prevent or reduces interspecific gene exchange (Scopece et al. 2013; Cahenzli et al. 2018) and an association between barrier number and introgressive gene flow has been reported (Shang et al. 2020). In these cases, rather than focusing on individual components of RI, disentangling the relative importance of multiple barriers is required to understand the potential fate of the hybrid zone.

Investigating RI in hybrid zones can also inform about the differentiation process because it allows to recognize the intensity of interspecific gene exchange (Baack et al. 2015; Vallejo-Marín and Hiscock 2016) and can allow to understand whether hybridization may have a "creative" role by generating new species (Mallet 2007). Each isolating trait may evolve at different rates, influencing how speciation proceed. Thus, depicting the rate of evolution of different isolating traits may clarify which barriers are important drivers of speciation and which accumulated after divergence (Coyne and Orr 2004). Several studies have shown a positive relationship between the strength of each barrier and genetic distance (reviewed in Coyne and Orr 2004). However, the rates of isolating barriers may vary substantially among different organisms. Certain groups of amphibians (Sasa et al. 1998), fish (Bolnick and Near 2005) and *Drosophila* (Turissini et al. 2018) show a clocklike accumulation of postzygotic RI, but more variable results are found in plants (reviewed by Baack et al. 2015). In this context, studies estimating different RI barriers across divergent taxa would increase our ability to generalize the role of reproductive barriers in speciation.

RI among closely related hybridizing species has been characterized for many Mediterranean members of the orchid family, arguably the second largest family in flowering plants after Asteraceae (Chase et al. 2016). These studies draw a picture in which multiple isolating barriers divide closely related species (Scopece et al. 2013; Scopece et al. submitted) and late postzygotic mechanisms (as hybrid sterility) arise prior to early postzygotic mechanisms as embryo mortality (Scopece et al. 2008). RI in this orchid group was found to be strongly influenced by pollination strategies of parental species (Cozzolino and Scopece 2008). For example, different hybridization scenarios are observed between species with

deceptive strategies with different levels of pollinator specialization (Cozzolino and Scopece 2008; Xu et al. 2011). Rewarding and rewardless flowers as food-deceptive orchids can also be hypothesized to experience different levels of pollinator sharing and thus of pollinator-mediated RI. In general, fruit set, pollination efficiency and flower constancy are lower in food-deceptive species, modifying the intensity of prezygotic barriers and increasing, consequently, the relative contribution of postzygotic barriers in preventing introgression (Scopece et al. 2010). On the other hand, rewarding species show higher levels of pollination efficiency due to an elevated flower constancy, which likely translates into more efficient prezygotic barriers. In such circumstances, postzygotic barriers can be weak due to the low incidence of heterospecific pollen flow, and introgression may occur due to fertile hybrids found in such hybrid zones (Natalis and Wesselingh 2012; Mota et al. 2019). In this Mediterranean orchid group, postzygotic barriers are thought to arise as a consequence of karyological changes (Cozzolino et al. 2004).

Even though by far most of orchid diversity is in the tropics, less is known for tropical members of the orchid family in terms of RI (but see Pinheiro et al. 2015). A notable exception is represented by the genus *Epidendrum* which arose in recent years as a model system to investigate reproductive isolation patterns in tropical orchids (Pinheiro and Cozzolino 2013). The description of RI mechanisms in this large genus is thus paramount to gain a picture on evolutionary patterns of the main clade of the orchid family (i.e., Epidendroideae). The genus *Epidendrum* is one of the biggest orchid groups in the neotropical region with approximately 1500 species (Hágsater and Soto Arenas 2005). Reward and rewardless species are found in *Epidendrum* (Cardoso-Gustavson et al. 2018), which is pollinated predominantly by Lepidoptera (Pinheiro and Cozzolino 2013). In this genus, several cases of hybridization have been reported (Pinheiro et al. 2010, 2015, 2016; Vega et al. 2013; Marques et al. 2014), suggesting the existence of weak pre-pollination barriers. Differently from Mediterranean orchids, hybrid zones between species with different karyotypes are still composed mostly by fertile hybrids, and postzygotic barriers are thought to be shaped by genetic incompatibilities (e.g., Pinheiro et al. 2010).

Most of the studies conducted on *Epidendrum* focused on species belonging to the subgenus *Amphyglottium*, which is predominantly composed of food-deceptive species (Cardoso-Gustavson et al. 2018). Hybrid zones with different levels of introgression and hybrid fertility have been found, ranging from contact zones with the predominance of F1 sterile hybrids (Pinheiro et al. 2015) to high levels of introgression mediated by abundant hybrid individuals (Pinheiro et al. 2010; Vega et al. 2013). These studies showed that pre and postzygotic barriers in this group may vary substantially, likely in consequence of extrinsic and intrinsic mechanisms found in each parental species pair, such as ploidy differences (Moraes et al. 2013; Vega et al. 2013; Marques et al. 2014), degree of pollinator specificity (Pinheiro et al. 2016), strong genetic incompatibilities (Pinheiro et al. 2015) and different habitat preferences (Pinheiro et al. 2010). A thorough investigation of different components of RI in a single study case is however still to be done.

In this study, we thus filled this gap by using a hybrid zone between *Epidendrum denticulatum* Barb. Rodr. and *Epidendrum orchidiflorum* Salzm. (Fig. 1) to investigate the contribution of different pre and postzygotic barriers in the genus *Epidendrum* and to understand the fate of the hybrid zone. The two parental species diverged more than 5 mya (Cardoso-Gustavson et al. 2018) and differ in a number of ecological traits. *E. orchidiflorum* is a rewarding species (Cardoso-Gustavson et al. 2018), which potentially attracts pollinators only at night (São Leão et al. 2019). In contrast, *E. denticulatum* is a food-deceptive species (Cardoso-Gustavson et al. 2018), which is visited primarily by different species of diurnal butterflies (Almeida and Figueiredo 2003; São Leão et al. 2019). Other

Fig. 1 Flower color polymorphisms found in *Epidendrum denticulatum* (**a**, **b**), *E.* x *purpureum* (**c**, **d**) and *E. orchidiflorum* (**e**, **f**) sampled at the hybrid zone studied in the Restinga de Massambaba population

floral attributes such as differences in flower color (Fig. 1) and odor emission (imperceptible for *E. denticulatum* and strong at night for *E. orchidiflorum*) were interpreted by São Leão et al. (2019) as important premating barriers which may prevent pollinator sharing between species. In addition, both species show a considerable divergence in habitat preferences (*E. denticulatum* occurs at higher densities in temporarily flooded shrubby vegetation, and *E. orchidiflorum* occurs in unflooded closed shrubby vegetation), as reported by São Leão et al. (2019). Despite all these differences, the hybrid between these species, *E.* x *purpureum*, was described in the same region where parental species co-occur (Rodrigues 1877), suggesting pollinator sharing to some extent.

Here, by disentangling the strength of seven different reproductive barriers, we aimed at answering the following questions:

(a) What is the contribution of pre- *versus* postzygotic RI mechanisms between *E. denticulatum* and *E. orchidiflorum*? Seven different barriers were calculated in order to understand the role of barriers acting on different life history stages of parental species and hybrids, such as habitat isolation, phenological isolation, embryo mortality and hybrid attractiveness;
(b) What karyotype differences and meiotic abnormalities may tell us about the fitness of hybrid plants? Detailed cytogenetic analysis was conducted on parental species and hybrids, aiming to quantify chromosome numbers and estimate meiotic abnormalities, which may influence late postzygotic barriers.

Materials and methods

Study area and plant material

Different patterns of geographic distribution are observed for *Epidendrum denticulatum* Barb. Rodr., which occur on Cerrado and coastal vegetation in southeastern Brazil (Pinheiro et al. 2015), and *Epidendrum orchidiflorum* Salzm., which prefers dry vegetation communities found in coastal and Caatinga vegetation in Northeastern Brazil (Pachon 2016). The distribution of both species overlaps along approximately 300 km in south eastern Brazil, along the seashore of Rio de Janeiro State, where *E. denticulatum* and *E. orchidiflorum* co-occur on coastal sand dune vegetation (Fig. S1). Sampling was conducted at Restinga de Massambaba (Cabo Frio–RJ, Fig. S1), where both parental species and their hybrid, *E.* x *purpureum*, are found in sympatry. We sampled plants at least 20 meters apart in order to avoid clones. Experiments were conducted with forty-five plants in total, which were cultivated for at least five years at Departamento de Biologia Vegetal, Instituto de Biologia, Universidade Estadual de Campinas (Campinas–SP).

Pollinia removal in parental and hybrid plants

According to São Leão et al. (2019), pollinia removal occurred in different periods for *E. denticulatum* (day) and *E. orchidiflorum* (night), suggesting complete premating barriers. In this experiment we aimed to test if pollinia removal may occur at different periods in both parental species, in a common garden environment. In addition, we also observed pollinia removal at day and night in *E.* x *purpureum*, to investigate if hybrids are able to attract diurnal and/or nocturnal potential pollinators. We used five specimens from each parental and hybrid plants, totalizing 15 individuals. Each plant had at least two inflorescences, and each flower stalk was exposed at different periods (day and night) to the insects. As we know that butterflies and moths are the only insects actively removing pollinia of *Epidendrum* species from subgenus *Amphyglottium* (Braga 1977; Almeida and Figueiredo 2003; Pinheiro and Cozzolino 2013; São Leão et al. 2019), we used nylon bags with very fine mesh to isolate the whole inflorescences. Before dawn, 15 inflorescences were unbagged and exposed to diurnal insects, and at the same time, the remaining 15 inflorescences were bagged to exclude diurnal visits. The opposite procedure was adopted before the sunset, where inflorescences exposed to diurnal insects were bagged, and those that remained bagged during the day were released to observe pollinia removal by nocturnal Lepidoptera. The total number of flowers in each inflorescence and the number of flowers without pollinia were recorded in each period (day and night) before bagging the inflorescences. Thus,

daytime and nighttime pollinia removal rates for each species were assessed by the number of flowers that had their pollinia partially or completely removed in each period over the average number of flowers exposed (P/F).

RI indices

All the RI indices were assessed based on data collected in the field (microhabitat and phenological isolation, hybrid habitat differentiation and hybrid attractiveness) or on experimental data obtained with cultivated plants (pollen-stigma incompatibility, embryo mortality and hybrid sterility). Microhabitat and phenological isolation indices were calculated using the data published by São Leão (2012) and São Leão et al. (2019), collected in the neighbor population of Maricá, within the same sand dune coastal vegetation formation where Restinga de Massambaba is situated. The remaining RI indices were calculated using data obtained from Restinga de Massambaba populations. To allow the comparison among different isolation mechanisms, we followed the method proposed by Sobel and Chen (2014). All the indices were thus calculated so that they can range between 0 (no isolation) and 1 (complete isolation). Below is a detailed description of each of the indices, ordered from the early acting to the late acting ones. The RI based on flower isolation was not included in our calculations because the identity of *E. orchidiflorum* pollinators is unknown.

Microhabitat isolation index ($RI_{MICROHABITAT}$)

We examined the microhabitat isolation index ($RI_{MICROHABITAT}$) between the species by quantifying the degree of co-occurrence of an area of 6 ha reported by São Leão et al. (2019). Plants were georeferenced and the obtained coordinates were used to construct a distribution map, published by São Leão et al. (2019). We subdivided the map reported by São Leão et al. (2019) into quadrats of 10 m × 10 m, and we counted the number of quadrats containing only *E. denticulatum*, only *E. orchidiflorum*, or both species. The proportion of quadrats that were shared and unshared for each species was determined, and from these proportions we calculated microhabitat isolation using equation RI_{4C} of Sobel and Chen (2014).

Phenological isolation index ($RI_{PHENOLOGY}$)

Phenological data were recorded weekly by São Leão (2012) during 1 year, between March 2010 and February 2011. Fourteen plants of each species were marked in the field and followed during the entire period, where the number of flowering plants was recorded in each visit (São Leão 2012). Estimation of the phenological isolation index ($RI_{PHENOLOGY}$) was carried out as described in Martin and Willis (2007) using the spreadsheet provided in Table S3 (Supporting information) of Lowry et al. (2008).

Pollen–stigma incompatibility ($RI_{POLLEN\text{-}STIGMA}$), embryo mortality ($RI_{EMBRYO\ MORTALITY}$) and hybrid sterility ($RI_{HYBRID\ STERILITY}$)

Three RI indices were calculated using data obtained from hand pollination using plants cultivated in a common garden. Manual crosses were conducted as described in Pinheiro

et al. (2010). Treatments were performed as follows: (i) self-pollinations using the two parental species and hybrids; (ii) cross-pollinations within species and hybrids; (iii) interspecific cross-pollinations; (iv) parental species acting as pollen donors for hybrid plants; (v) hybrids acting as pollen donors for parental species. All crosses were conducted in both directions; each plant provided and received pollen. In order to avoid abortions due to an overload of fruits on each inflorescence, a limit of four fruits by inflorescence was adopted, based on previous results of crossing experiments using *Epidendrum* species (Pinheiro et al. 2010, 2015). In total, 152 flowers were used from 32 plants (13 *E. denticulatum*, 7 *E. orchidiflorum* and 12 hybrids).

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 200 seeds from each fruit were analyzed under the microscope. The percentage of viable seeds was calculated by dividing the number of viable embryos by the total number of embryos scored. Fruit and seed data were obtained for each cross type and compared using Mann–Whitney or Kruskal–Wallis tests with SPSS 11.0 software.

The strength of three postmating individual barriers were estimated following Scopece et al. (2013): pollen–stigma incompatibility ($RI_{\text{POLLEN-STIGMA}}$), embryo mortality ($RI_{\text{EMBRYO MORTALITY}}$) and hybrid sterility ($RI_{\text{HYBRID STERILITY}}$). Because most crosses were bidirectional, individual barriers were calculated for each parental species (*E. denticulatum* and *E. orchidiflorum*) when acting as pollen recipients. Fruit set results were used to estimate the strength of pollen–stigma incompatibility, and embryo viability was estimated using seed viability measures. The individual barriers based on pollen–stigma compatibility and seed viability were calculated using equation RI_{4A} following Sobel and Chen (2014).

Fruit set and seed viability obtained from backcrosses (parental species × hybrids) were considered two stages of hybrid sterility. Thus, the strength of hybrid sterility was calculated separately for each stage. Individual barriers were calculated as described above, but using a modified version of the equation RI_{4A} (Sobel and Chen 2014) following Pegoraro et al. (2016). The strength of hybrid sterility based on the fruit set stage was defined as $RI_{\text{hybrid\_F}} = 1 - 2 * (\%\ \text{fruit formed in backcross} / \%\ \text{fruit formed in backcross} + \text{the maximum hypothetical fruit set})$. Similarly, the strength of hybrid viability based on the seed viability stage was defined as $RI_{\text{hybrid\_S}} = 1 - 2 * (\%\ \text{seed viability in the backcross} / \%\ \text{seed viability in the backcross} + \text{the maximum hypothetical seed viability})$. In both cases, the maximum hypothetical fruit set and seed viability values were equal to one. Following Pegoraro et al. (2016), $RI_{\text{hybrid\_F}}$ and $RI_{\text{hybrid\_S}}$ were combined for all of 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 indexes was used to calculate the total strength of hybrid viability ($RI_{\text{HYBRID STERILITY}}$) for each parental species acting as pollen recipient.

Hybrid habitat differentiation index ($RI_{\text{HYBRID HABITAT DIFFERENCE}}$)

Hybrid persistence in contact zones may be facilitated when hybrids occur in habitats which differ from those occupied by parental species. The persistence of hybrid plants

may impact the RI between parental species because hybrids may act as bridges to gene exchange. To explore this extrinsic postzygotic mechanism, we estimated the number of 100 m^2 quadrats where the hybrid coexists with both the parental species (heterospecific quadrats), the number of quadrats in which the hybrid occurs with only one of the parental species (semi-heterospecific quadrats) and the number of quadrats in which only the hybrid occurs (conspecific quadrats). $RI_{HYBRID\ HABITAT\ DIFFERENCE}$ was calculated as: 1 − (number of heterospecific quadrats/number of heterospecific quadrats + number of semi-heterospecific quadrats + number of conspecific quadrats).

Hybrid attractiveness ($RI_{HYBRID\ ATTRACTIVENESS}$)

Hybrid phenotypes may have reduced pollination attraction, thus limiting the gene exchange between parental species (Scopece et al. 2013). Natural pollination success was estimated in the field as the fruit-flower ratio of parental species and hybrids in 2019. We calculated an extrinsic postzygotic isolation index comparing natural pollination success (i.e. the number of fruits produced relative to the total number of flowers in an inflorescence) of hybrids versus parental species. This index was calculated following Scopece et al. (2013).

Relative strength and absolute contribution of different isolation mechanisms

The seven indexes described above ($RI_{MICROHABITAT}$, $RI_{PHENOLOGY}$, $RI_{POLLEN\text{-}STIGMA}$, $RI_{EMBRYO\ MORTALITY}$, $RI_{HYBRID\ STERILITY}$, $RI_{HYBRID\ HABITAT\ DIFFERENTIATION}$ and $RI_{HYBRID\ ATTRACTIVENESS}$) 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). To compare the contribution of premating versus post-mating and of prezygotic versus postzygotic mechanisms, following Pegoraro et al. (2016), we calculated indices of total isolation for each barrier category (i.e. pre-mating: RI_{PREM_TOT}; post-mating: RI_{POSTM_TOT}; prezygotic: RI_{PREZ_TOT}; postzygotic: RI_{POSTZ_TOT}).

Cytogenetic analyses

To evaluate the normality of the pollen meiotic process, which can be considered a proxy of male fertility, two plants of *E. denticulatum*, one plant of *E. orchidiflorum* and two hybrids were analyzed. 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, 5 min each, in distilled water, digested in 1% (w/v) macerozyme (Sigma, St Louis, MO, USA), 2% (w/v) cellulase (Onozuka, St Louis, MO, USA) and 20% (v/v) pectinase (Sigma, St Louis, MO, USA) solution at 37 °C for 5 min, and squashed in a drop of 60% acetic acid. The slides were frozen in liquid nitrogen and stained using a solution of 1:1 (v/v) Vectashield® with DAPI. 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. The average frequency of meiotic abnormality (cells carrying out an abnormal meiosis) among the parental species and hybrids were compared using the Kruskal–Wallis tests with SPSS 11.0 software.

Mitotic analyses were performed using the same plants sampled for meiosis. Root tips were pretreated with 0.002 M 8-hydroxyquinoline (Sigma, St Louis, MO, USA) 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 1% (w/v) macerozyme (Sigma), 2% (w/v) cellulase (Onozuka) and 20% (v/v) pectinase (Sigma) solution for 30 min at 37 °C, squashed in a drop of 60% acetic acid and frozen in liquid nitrogen. The best slides were stained using a solution of 1:1 (v/v) Vectashield® with DAPI (1 mg μl^{-1}; Sigma, St Louis, MO, USA). 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.).

Analyses of flow cytometry followed Pinheiro et al. (2016), and a total of nine individuals were used in this analysis, three plants from each species and hybrids. 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) 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. Flow cytometry was performed on a BD Biosciences FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA), and data were collected using CellQuestPro software (Becton-Dickinson, San Jose, CA, USA). 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.

Results

Diurnal and nocturnal pollinia removal

Observations were made during 33 days, between 21 May and 28 June 2019, during the dry season, where the average temperature in the period was 19.9 °C (CEPAGRI, UNICAMP). In total, 30 inflorescences and 826 flowers were monitored during the period of the experiment (Table 1). Pollinia removal during the day and at night were observed for both parental species and for hybrids. For *E. denticulatum*, diurnal pollinia removal was significantly higher than nocturnal pollinia removal (N = 10, U = 0.000, $P < 0.01$), which showed the lowest value recorded in the whole experiment (P/F = 0.03 ± 0.05 SD). Diurnal pollinia removal in *E. denticulatum* also returned significantly higher values when compared to *E. orchidiflorum* (N = 10, U = 0.000, $P < 0.01$) and hybrids (N = 10, U = 2.000,

Table 1 Daytime (D) and nighttime (N) pollinia removal rates for each species and hybrid plants, including the number of flowers observed (FL) during the whole period (33 days), number of pollinia removed (PO) and the number of pollinia removed over the number of observed flowers (P/F)

Experiment	FL	PO	P/F (SD)[1]
E. denticulatum (D)	152	89	0.58 (0.09)a
E. denticulatum (N)	150	5	0.03 (0.05)b
E. orchidiflorum (D)	119	9	0.07 (0.09)b
E. orchidiflorum (N)	118	9	0.07 (0.06)b,d
E. x *purpureum* (D)	146	52	0.35 (0.15)c
E. x *purpureum* (N)	141	27	0.18 (0.08)c,d
Total	826		

[1]The same letter indicates that the means are not significantly different ($P > 0.05$)

$P < 0.05$). Similar diurnal and nocturnal pollinia removal were observed in *E. orchidiflorum* (day P/F = 0.07 ± 0.09 SD, night P/F = 0.07 ± 0.06 SD). When compared to the parental species, *E.* x *purpureum* showed intermediate values of pollinia removal during the day (P/F = 0.35 ± 0.15 SD) and night (P/F = 0.18 ± 0.08 SD). Pollinia removal during the day in hybrid plants was significantly higher than values observed in *E. orchidiflorum* (N = 10, U = 2.000, $P < 0.05$). During the night, significant higher values of pollinia removal were observed in *E.* x *purpureum* when compared to *E. denticulatum* (N = 10, U = 1.000, $P < 0.05$).

Strength of prezygotic barriers

Considering the 600 quadrats of 10 m × 10 m analyzed (6 ha in total), parental species were found in only 67 quadrats. The co-occurrence of parental species was recorded in 14 quadrats, and the remaining 53 quadrats were occupied only by *E. denticulatum* (19) or *E. orchidiflorum* (34). Thus, $RI_{MICROHABITAT}$ was 0.57 for *E. denticulatum* and 0.70 for *E. orchidiflorum* (Table 2).

Both parental species show a broad overlap in flowering time. According to São Leão (2012), specimens of *E. denticulatum* and *E. orchidiflorum* produce flowers in all months, showing a flowering peak between December and February (Figs. 27 and 28 of São Leão 2012). Indeed, a weak $RI_{PHENOLOGY}$ was found for *E. denticulatum* (0.04) and *E. orchidiflorum* (0.06, Table 2).

Most intraspecific pollinations resulted in mature fruits with no significant differences among all comparisons (Table 3). The $RI_{POLLEN\text{-}STIGMA}$ was 0.06 for *E. denticulatum* and 0.19 for *E. orchidiflorum* (Table 2).

Strength of postzygotic barriers

Seed viability returned variable results depending on the crossing type. Intraspecific crosses returned similar results for E. *denticulatum* (0.80 ± 0.15 SD) and *E. orchidiflorum* (0.83 ± 0.08 SD). However, a significant decrease (N = 23, U = 17.500, $P < 0.01$) in seed viability was detected in *E. orchidiflorum* when comparing cross and self-pollinations. Interspecific crosses, where *E. denticulatum* acted as seed parent and pollen donor to *E. orchidiflorum*, returned significant differences (N = 12, U = 0.000, $P < 0.01$), with a 21% decrease in seed viability when *E. orchidiflorum* acted as pollen donor to *E. denticulatum*.

Table 2 Estimates of the relative strength (RS) and absolute contribution (AC) of reproductive isolation (RI) between *E. denticulatum* and *E. orchidiflorum*, considering three prezygotic barriers (microhabitat isolation, phenology, pollen-stigma incompatibility) and four postzygotic barriers (embryo mortality, hybrid sterility, hybrid habitat difference and hybrid attractiveness)

Species	Premating						Post-mating								Total isolation
	Prezygotic						Intrinsic postzygotic				Extrinsic postzygotic				
	$RI_{MICROHABITAT}$		$RI_{PHENOLOGY}$		$RI_{POLLEN\text{-}STIGMA}$		$RI_{EMBRYO\ MORTALITY}$		$RI_{HYBRID\ STERILITY}$		$RI_{HYBRID\ HABITAT\ DIFFERENCE}$		$RI_{HYBRID\ ATTRACTIVENESS}$		
	Str	AC	Str	AC	Str	AC	Str	AC	Str	AC	Str	AC	Str	AC	
E. denticulatum	0.57	0.57	0.04	0.01	0.06	0.04	0.08	0.04	0.23	0.10	–	–	–	–	0.79
E. orchidiflorum	0.70	0.70	0.06	0.01	0.19	0.08	−0.01	0.00	0.25	0.07	–	–	–	–	0.97
E. x *purpureum*	–		–		–		–		–		0.00	0.00	0.44	0.10	
Total	0.64	0.64	0.05	0.02	0.13	0.07	0.03	0.01	0.24	0.09	0.00	0.00	0.44	0.10	0.93
Asymmetry	0.13		0.02		0.13		0.09		0.02						0.18

All measures of isolation varied between 0 (no isolation) and 1 (complete isolation)

Table 3 Fruit formation and viable seeds produced from hand pollination of *Epidendrum denticulatum*, *E. orchidiflorum* and *E.* x *purpureum*, 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)[2]	SV (SD)[2]
Intraspecific crosses						
E. denticulatum (7)	*E. denticulatum* (7)	7	15	15	1.0000 (0.00)a	0.8050 (0.15)a,e
E. orchidiflorum (5)	*E. orchidiflorum* (7)	7	14	13	0.9286 (0.27)a	0.8346 (0.08)a
Self pollinations						
E. denticulatum (3)	–	3	11	11	1.0000 (0.00)a	0.7009 (0.12)d,e,f
E. orchidiflorum (5)	–	5	10	10	1.0000 (0.00)a	0.5435 (0.29)b,f,g
Interspecific crosses						
E. denticulatum (5)	*E. orchidiflorum* (2)	7	8	7	0.8750 (0.35)a	0.6879 (0.09)b,e
E. orchidiflorum (4)	*E. denticulatum* (4)	8	8	5	0.6250 (0.52)a	0.8680 (0.03)a
Backcrosses with *E. denticulatum*						
E. denticulatum (7)	*E.* x *purpureum* (4)	11	14	13	0.9286 (0.27)a	0.6835 (0.16)b,f
E. x *purpureum* (6)	*E. denticulatum* (7)	13	18	18	1.0000 (0.00)a	0.6036 (0.23)b,f
Backcrosses with *E. orchidiflorum*						
E. orchidiflorum (3)	*E.* x *purpureum* (3)	6	9	8	0.8889 (0.33)a	0.6988 (0.34)a,e,f
E. x *purpureum* (4)	*E. orchidiflorum* (4)	8	11	10	0.9091 (0.30)a	0.6430 (0.23)b,e
Crosses using hybrids only						
E. x *purpureum* (7)	*E.* x *purpureum* (7)	7	20	15	0.7500 (0.44)a	0.2980 (0.30)c,g
E. x *purpureum* self-pollination (5)	–	5	14	12	0.8571 (0.36)a	0.2372 (0.17)c
Total		32	152	137		

[1]Total number of plants used

[2]The same letter indicates that the means are not significantly different ($P > 0.05$)

$RI_{EMBRYO\ MORTALITY}$ was low for both parental species (*E. denticulatum* = 0.08, *E. orchidiflorum* = −0.01, Table 2).

A significant decrease in seed viability (N = 77, U = 343.000, $P < 0.001$) was observed when comparing pooled intraspecific crosses (0.81 ± 0.11 SD) and backcrosses, when both parental species acted as pollen recipients and donors to hybrid plants (0.64 ± 0.23 SD). 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 considering comparisons between *E.* x *purpureum* and *E. denticulatum* (N = 31, U = 88.000, $P = 0.258$) and between *E.* x *purpureum* and *E. orchidiflorum* (N = 18, U = 25.000, $P = 0.203$). Values of $RI_{HYBRID\ STERILITY}$ were the third strongest among all barriers tested (*E. denticulatum* = 0.23, *E. orchidiflorum* = 0.25, Table 2).

In the area of Restinga de Massambaba, hybrids were found on five plots of 100 m^2, and in all of them both parental species were also present. Therefore, RI due to hybrids occurring in different habitats was absent ($RI_{HYBRID\ HABITAT\ DIFFERENTIATION} = 0.00$).

We estimated natural pollination success of 34 individuals (13 *E. denticulatum*, 12 *E. orchidiflorum* and nine hybrids, Table 4). The value of fruit-flower ratio observed for *E.*

Table 4 Estimates of natural pollination success based on fruit set and pollinia removal at population of Restinga de Massambaba, where *E. denticulatum*, *E. orchidiflorum* and *E.* x *purpureum* co-occur, including sample size (N), number of flowers (FL), number of fruits (FR), the ratio of fruits over the number of flowers (FR/FL), the number of flowers with pollinia removed (PR), and the ration of pollinia removed over the number of flowers (PR/FL)

Species (N)	FL	FR	FR/FL	PR	PR/FL
E. denticulatum (13)	925	6	0.006	6	0.006
E. orchidiflorum (12)	416	13	0.031	7	0.016
E. x *purpureum* (9)	825	6	0.007	5	0.006

orchidiflorum (3.13%) was almost four times higher than values observed for *E. denticulatum* and hybrids (0.65% and 0.73%, respectively). The $RI_{HYBRID\ ATTRACTIVENESS}$ was 0.44 (Table 2).

Self and cross hand pollinations performed only with *E.* x *purpureum* plants showed an average fruit formation of 79.4%. Despite the similarity in average seed viability found between self (0.23 ± 0.17 SD) and cross pollinations (0.35 ± 0.30 SD) of hybrid plants (Table 3), results in each experiment were highly variable, ranging from 0.0 to 59.0% in self-pollinations, and 0.0–95.0% in crosses between different hybrid plants.

The individual effects of the two premating barriers and five post-mating barriers are summarized for each species in Table 2. The cumulative effect of these barriers nearly reached unity for *E. denticulatum* (0.79) and *E. orchidiflorum* (0.97). Given the high values of microhabitat isolation observed for both parental species, post-mating barriers made little contribution to species isolation (Table 2). On the other hand, phenological isolation and embryo mortality were the weakest barriers observed. Hybrid attractiveness was the stronger post-mating and postzygotic RI between parental species, with a strength of 0.44 (Table 2). The absolute contribution of premating mechanisms (0.65) were comparable to post-mating mechanisms (0.70), and a similar trend was also observed when comparing pre (0.72) and postzygotic mechanisms (0.63, Fig. S2).

Meiotic and mitotic analyses

The meiotic analysis of *E. denticulatum* and *E. orchidiflorum* found high levels of meiotic normality, with an average of 90.9% and 85.5% respectively (Table 5). Meiotic cells in both parental species showed chromosomes pairing as bivalents and following meiotic division forming four equal cells. In contrast, the meiotic analyses of hybrids found significantly higher levels of abnormalities when compared to parental species ($\chi^2 = 17.353$, $df = 4$, $P < 0.01$), with 55.8%, in average, of cells carrying out normal meiosis. Chromosome outside the equatorial plate in metaphase I/II (early and late disjunction of bivalents/chromosomes), laggard chromosomes and chromatin bridges in anaphase I/II and micronuclei formation in telophase II were the most common abnormalities observed in hybrids (Fig. 2). Triads, tetrads, and polyads with irregular microcytes were also observed.

The chromosome number during meiosis could be determined only for *E. denticulatum* ($n = 26$) and *E.* x *purpureum*, in which $n = 52$ was the prevalent number, with few cells showing $n = 26$. In accordance with the meiotic counts, mitotic metaphases from *E. denticulatum* and *E. orchidiflorum* presented, respectively, $2n = 52$ and $2n = 156$ (Table S1, Fig. S3). The hybrid specimens presented multiple numbers ranging from $2n = 104$ to

Table 5 Meiotic behavior of *Epidendrum denticulatum*, *E. orchidiflorum* and *E.* x *purpureum* in the following division phases: prophase I (P.I.), meiosis I (M.I.), anaphase I (A.I.), telophase I (T.I), prophase II (P.II.), meiosis II (M.II), anaphase II (A.II), telophase II (T.II) and tetrad (Tetr.), including the number of abnormal cells found in each phase (proportion of the total), the total number of cells analyzed (N), and the total percentage of abnormal cells (Ab)

Species	Meiosis I				Meiosis II					N	Ab (%)[1]
	P. I.	M. I.	A. I.	T. I.	P. II.	M. II.	A. II.	T. II.	Tetr.		
E. denticulatum	0 (0.00)	1 (0.5)	–	–	–	–	–	–	6 (0.13)	54	12.9
E. denticulatum	1 (0.03)	6 (0.09)	0 (0.00)	–	0 (0.00)	–	–	–	–	132	5.3
E. orchidiflorum	0 (0.00)	0 (0.00)	0 (0.00)	–	1 (0.04)	3 (0.03)	18 (0.25)	4 (0.10)	3 (0.17)	200	14.5
E. x *purpureum*	2 (0.10)	4 (1.00)	6 (0.85)	2 (0.14)	3 (0.30)	16 (0.48)	4 (0.22)	11 (0.47)	0 (0.00)	129	37.2
E. x *purpureum*	24 (0.60)	–	1 (1.00)	–	5 (0.38)	18 (0.60)	8 (0.88)	7 (0.35)	45 (0.45)	211	51.1

[1]The same letter indicates that the means are not significantly different ($P > 0.05$)

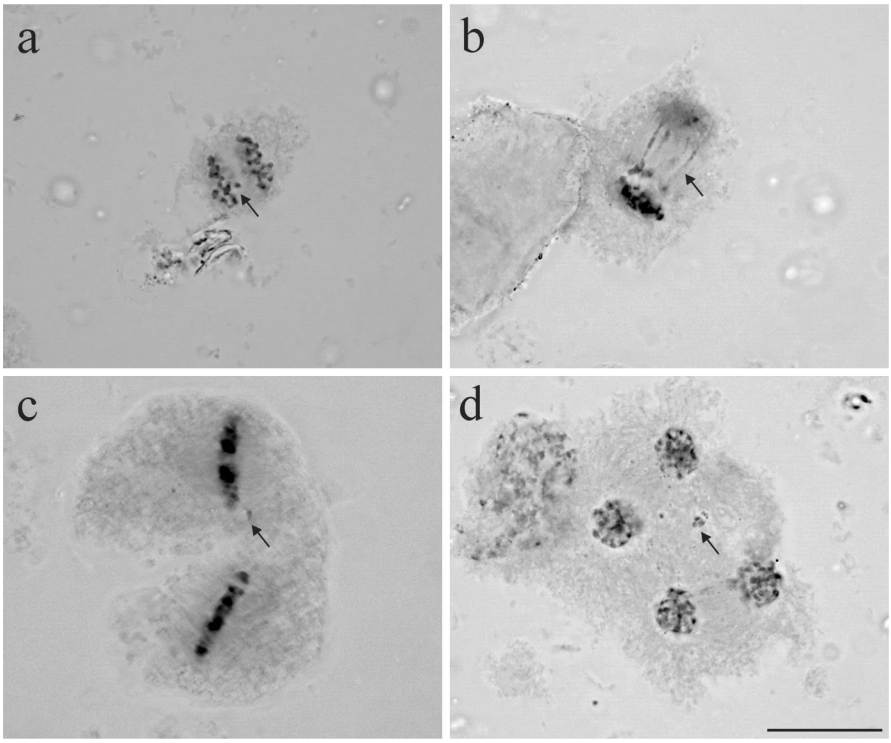

Fig. 2 Main meiotic abnormalities, indicated by the black arrow, observed in *E.* x *purpureum*: **a** Chromosome outside the equatorial plate in metaphase I (early disjunction of bivalents); **b** Chromatin bridges in anaphase I; **c** Chromosome outside the equatorial plate in metaphase II; **d** Telophase II with laggard chromosomes migration. Scale bar in d indicates 20 μm

2n = 106 (Fig. S3), and were the same individuals where meiotic irregularities were detected. In addition, polyploidization was not observed in any hybrid plant. The mean genome size estimated in parental species was 4.66 ± 0.09 pg/2C in *E. denticulatum* and 8.15 ± 0.20 pg/2C in *E. orchidiflorum*. The hybrids genome size ranged from 6.13 to 6.39 pg/2C. The genome size of *E. denticulatum* was significantly lower than values observed in *E. orchidiflorum* and hybrids ($\chi^2 = 7.200$, $df = 2$, $P < 0.05$, Table S1).

Discussion

Disentangling the role of different isolating mechanisms in hybrid zones is mandatory in order to gain information on the fate of divergence between parental species. Here we analysed seven different pre and postzygotic mechanisms between two neotropical orchid species *E. denticulatum* and *E. orchidiflorum*. Overall, we found that prezygotic isolation is mainly due to habitat diversification and that postzygotic mechanisms are permeable despite dramatic karyological changes. Taken together, they surprisingly show a picture of a strong but incomplete isolation between these two well-divergent species with the presence of fertile hybrids.

Prezygotic isolating mechanisms

Among prezygotic barriers, the contribution of habitat isolation accounts for more than half of the total strength observed between species, suggesting that adaptation to contrasting environmental conditions between species (São Leão et al. 2019) may be the main driver of RI (Table 2). *Epidendrum* parental species and hybrids are found in close proximity in open unflooded shrubby vegetation, but important differences in habitat preferences were also observed (São Leão et al. 2019). For instance, only *E. denticulatum* is found in open and temporarily flooded shrubby vegetation. In contrast, *E. orchidiflorum* and hybrids occur in unflooded closed shrubby vegetation, where *E. denticulatum* is not found (São Leão et al. 2019). The adaptation of plants to different environments has been understood as an important reproductive isolating barrier (reviewed by Baack et al. 2015). For example, differences in soils and temperature may translate into effective barriers to gene exchange between sympatric species (Lexer et al. 2003; Abbott and Brennan 2014; Hipperson et al. 2016). In such cases, parental genotypes would have lower probabilities of establishing in the alternative habitat, leading to a RI based on immigrant inviability (Nosil et al. 2005). Microhabitat isolation detected between *E. denticulatum* and *E. orchidiflorum* suggests that flooding and shading tolerance may act as important selective forces shaping specific ecological traits in each parental species. In fact, different ecological traits are associated with flood and shade tolerance, drastically changing morphological and physiological responses in roots, stems and leaves. Flooding tolerance involves synergies among traits for improved internal aeration, anoxia tolerance and recovery, both for roots during soil waterlogging and shoots during submergence (Colmer and Voesenek 2009). On the other hand, high levels of plasticity in leaf morphological features optimizing light capture, are expected in shade tolerant species (Valladares and Niinemets 2008). Indeed, high levels of habitat isolation have been found in hybrid zones composed of species with contrasting responses to flood and shade. Martin et al. (2006) studied a hybrid zone during a seasonal event of flood and detected higher survival rates in the flood-tolerant *Iris fulva* than its congener adapted to dry habitats, *I. brevicaulis*. A gradient from high light levels to shady sites segregates pure parental genotypes of two hybridizing fern species, shaping the genetic architecture of this hybrid zone (Kentner and Mesler 2000). The evolution of different habitat preferences between *E. denticulatum* and *E. orchidiflorum* probably occurred in allopatry since both species are currently distributed in different regions (Pinheiro et al. 2015; Pachon 2016).

Prezygotic isolation in orchids is traditionally considered to be related to the highly specialized pollination strategies (Armbruster and Muchhala 2009). However, pollination strategies are extremely variable in the orchid family and are often generalized allowing for intense pollinator sharing and thus weak pollinator-mediated reproductive isolation (Cozzolino et al. 2005; Jersáková et al. 2006). Here we investigated isolating mechanisms between two *Epidendrum* species with different pollination strategies (the rewarding night pollinated *E. orchidiflorum* and the food-deceptive daily-pollinated *E. denticulatum*). Contrarily to our expectations, our data on pollinia removal suggest a weak role for pollinator isolation. We have not directly estimated this barrier but we found that hybrid and parentals attract pollinators both during the day and during the night thus suggesting some extent of pollinator sharing. Despite the mix of parental genomes with very different adaptations, hybrid plants are still able to attract pollinators and show a pollination success similar to one of the parental species, the food-deceptive

E. denticulatum. In addition, our experiment showed pollinia removal in hybrid plants during day and night, suggesting a flexible pollination system where flowers emit signals able to attract diurnal and nocturnal pollinators.

Phenological isolation is another important RI barrier between closely related orchid species (Pegoraro et al. 2016). The potential for this barrier in tropical environments is elevated, as opposed to zones with temperate climates where flowering displacement is constrained by seasonality. In the present study case, however, phenological isolation was very weak and was responsible only for a modest reduction of gene flow. This low contribution is due to the large overlap in flowering time observed between *E. denticulatum* and *E. orchidiflorum* (Table 2), which produce flowers almost the entire year (São Leão 2012). Pollen stigma incompatibility, the last prezygotic mechanism we investigated, was found to contribute weakly to RI (0.069 *E. denticulatum*, 0.194 *E. orchidiflorum*). This barrier was found to be important in keeping species boundaries in groups that are more likely to experience pollinator sharing (Scopece et al. 2007).

Variable intensities in postzygotic barriers

Given that *E. denticulatum* and *E. orchidiflorum* are not sister or closely related species, we expected an important contribution of postzygotic mechanisms. We found that postzygotic barriers contributed to RI with different intensities in the two parental species (Table 2). Considering the intrinsic postzygotic barriers studied here, embryo mortality was less effective in limiting gene exchange than hybrid sterility, which showed higher and similar values for both parental species. A significant decrease in seed viability was observed when comparing intraspecific crosses and backcrosses, in which hybrids acted as pollen recipients and donors to parental species. The large differences in chromosome numbers and genome sizes detected between parental species (Table S1) may have affected hybrids reproduction by increasing meiosis abnormalities (Table 5). This result is evident in the crosses including only hybrids, which showed a mean seed viability of 29% (Table 3). Differences in chromosome numbers have been considered instantaneous barriers to gene exchange (Coyne and Orr 2004), mainly because interploidy crosses often result in strong hybrid inviability and sterility (Köhler et al. 2010). According to Levin (2012), a pronounced reduction in hybrid fertility tends to appear after lineages have been separate for more than 4 million year, which is less than the divergence time of 5 million year observed between E. *denticulatum* and *E. orchidiflorum* (Cardoso-Gustavson et al. 2018). Thus, we believe most genetic incompatibilities and chromosome changes between parental species may have occurred within this time interval. Hybrid sterility made a substantial contribution among the post-mating isolating mechanisms, which is potentially connected to the large differences in chromosome number found between parental species (*E. denticulatum* 2n – 52, *E. orchidiflorum* 2n = 140). Several meiotic abnormalities were found in hybrid plants, decreasing the seed viability of crosses where *E.* x *purpureum* acts as pollen donor and receptor. Despite the meiotic problems and low fertility found in crosses including hybrids, the values of seed fertility are highly variable, mainly in crosses between hybrid plants, suggesting hybrids with different genetic background and potentially different hybrid classes (F1, F2, backcrosses), a similar result was found in other *Epidendrum* hybrid zones (Pinheiro et al. 2010, 2016).

The two extrinsic postzygotic barriers analyzed in this study returned contrasting results. Hybrids were always found growing together with parental species, indicating that RI associated with *E.* x *purpureum* exploring different habitats was absent

($RI_{HYBRID\ HABITAT\ DIFFERENCE} = 0$, Table 2). On the other hand, $RI_{HYBRID\ ATTRACTIVENESS}$ was the strongest postzygotic barrier found (Table 2). This barrier was strongly influenced by differences in natural pollination success observed between parental species and hybrids (Table 4). Pollination success was almost five times higher in *E. orchidiflorum* than *E. denticulatum* and *E.* x *purpureum*, and this strong difference is probably related to the presence of nectar only in *E. orchidiflorum* flowers. Nectar secreting species show higher levels of pollination success than food-deceptive species (Tremblay et al. 2005; Scopece et al. 2010). The number of pollinator visits and their flower constancy to rewarding plant species increase pollination success and overall pollination efficiency (Tremblay et al. 2005; Scopece et al. 2010). In contrast, food-deceptive species are less visited by pollinators, which also show low specificity for particular plant species (Cozzolino et al. 2005), increasing pollen loss or heterospecific pollen deposition (Scopece et al. 2010). Similar results were reported by Ren et al. (2014), confirming that contrasting pollination strategies may influence RI mechanisms and the structure of hybrid zones.

Relative importance of pre and postzygotic mechanisms

The apparent dominance of prezygotic over postzygotic mechanisms may restrict our ability to interpret barriers of gene exchange acting in later phases. According to Martin and Willis (2007), isolation mechanisms have a sequential nature, which gives the prezygotic barriers a greater effect than postzygotic barriers, even if the different mechanisms have an equivalent strength. Lowry et al. (2008) suggests that collapsing individual barriers into categories based on the timing of mechanisms (pre- or postzygotic) may reduce the bias in interpreting the relevance of pre and postzygotic barriers. Following this procedure, we found both pre and postzygotic mechanisms act to prevent gene flow between parental species. In *E. denticulatum*, the strongest effect of prezygotic barriers was confirmed since postzygotic mechanisms (0.308) have nearly half the strength of prezygotic mechanisms (0.637). However, in *E. orchidiflorum* similar values were found for both pre (0.806) and postzygotic mechanisms (0.754). Contrary to predictions, we found postzygotic barriers to be more variable than prezygotic barriers, a potential effect of the strong genetic differences found between parental species, expressed here as divergent chromosome numbers and genome size (Table S1, Fig. S3).

Consistent with previous studies (reviewed by Baack et al. 2015), we found prezygotic barriers contribute more to total RI. Studies investigating the evolution of RI mechanisms have shown prezygotic barriers appearing sooner and developing at faster rates than postzygotic barriers (Scopece et al. 2007; Lowry et al. 2008; Turissini et al. 2018). It is expected that natural selection has a primary role in acting upon prezygotic isolation mechanisms, such as divergent habitat preferences and floral attractants, accelerating the evolution of such ecological traits (Schemske 2010; Levin 2012). In contrast, the intensity of postzygotic mechanisms is related to the gradual stochastic accumulation of many genetic incompatibilities and chromosomal rearrangements (Orr and Turelli 2001; Scopece et al. 2008). Consequently, the time required for the formation of postzygotic barriers may be several orders of magnitude longer than the time required for prezygotic isolation (Scopece et al. 2007). Thus, the strong difference in strength between pre and postzygotic mechanisms observed in *E. denticulatum* and *E. orchidiflorum* may be related to the ecological divergence observed between the species, reflected by contrasting habitat preferences. Several studies have shown this asymmetric pattern of strength between pre and post-mating barriers (reviewed by Baack et al. 2015), and similar asymmetry is found between pre and

postzygotic mechanisms (Nosil et al. 2005; Martin and Willis 2007; Lowry et al. 2008, Paudel et al. 2018).

What could be the fate of *E. x purpureum*?

Hybrid plants showed lower reproductive success than parental species in crossing experiments (Table 3) and higher meiotic behavior abnormalities (Table 5), which made a substantial contribution to the RI observed between *E. denticulatum* and *E. orchidiflorum*. Hybrids' reproductive failure may be interpreted as a direct product of Bateson-Dobzhansky-Muller genic incompatibilities (Orr and Turelli 2001), transposable element repressor mismatches (Serrato-Capuchina and Matute 2018) and differences in chromosome number and genome size observed between parental species (Table S1, Fig. S3). Differences in ploidies and genome size have been traditionally recognized as instantaneous barriers to gene exchange due to the extensive abnormalities in chromosome pairing, meiosis and low fitness found in interploidy hybrids (reviewed by Marques et al. 2018). However, a closer inspection of the results revealed highly variable levels of fertility among hybrid plants, suggesting some individuals have overcome the ploidy barrier. In fact, a number of studies have shown that interploidy crosses may result in viable odd-ploidy offspring (Burton and Husband 2000; Chapman and Abbott 2010; Vallejo-Marín et al. 2016), including *Epidendrum* species (Pinheiro et al. 2010; Moraes et al. 2013; Vega et al. 2013; Marques et al. 2014).

While hand pollination experiments show low levels of hybrid fertility, natural pollination success show a different picture. Despite the fact that natural pollination success was much higher in the rewarding parental species (*E. orchidiflorum*), *E. denticulatum* and *E.* x *purpureum* showed lower but similar values (Table 4). According to Cardoso-Gustavson et al. (2018) *E.* x *purpureum* does not offer any reward to pollinators as *E. denticulatum*, suggesting a similar pollination strategy based on generalist pollinators. We have no information regarding the identity of *E.* x *purpureum* pollinators, and field observations are crucial to understand which insects visit hybrid plants. However, considering that pollinia removal occurred during the day and night in hybrid plants (Table 1), we may expect that *E.* x *purpureum* is visited by butterflies and moths which also visit *E. denticulatum* and *E. orchidiflorum*. Parental species and *E.* x *purpureum* belong to subgenus *Amphyglottium*, a clade characterized by the generalist nature of pollination strategies, in which most species are food-deceptive (reviewed by Pinheiro and Cozzolino 2013). In fact, food-deception appears to be a stable evolutionary strategy in *Epidendrum* because significant asymmetric transitions to food-deception, from ancestral rewarding species, were detected (Cardoso-Gustavson et al. 2018).

Our results do not support any ecological segregation of hybrid plants. *E.* x *purpureum* co-occurs with parental species in the same habitats, and potentially explores the same pollinators. In addition, the frequency of hybrid plants in the field is approximately 20 times lower than parental species (Pinheiro et al., unp. res.). Thus, at present, there is no evidence that *E.* x *purpureum* can be regarded as a hybrid species. Hybrids emerge in parental populations and must overcome competition with parental species in order to avoid genetic blurring through backcrossing (Coyne and Orr 2004). In such a scenario, ecological segregation may play an important role in hybrid speciation because the colonization of new habitats and ecological niches would decrease the competition with parental taxa (Marques et al. 2016; Vallejo-Marin et al. 2016). Regardless of the outcome of this debate, the persistence of *E.* x *purpureum* is favored by a diverse array

of ecological traits, such as asexual and sexual reproduction, perenniality, and high levels of fertility found in some individuals, as reported for other plant species (Rieseberg and Willis 2007). Hybridization can impact different developmental and physiological traits, leading to shifts in ecological tolerances of hybrids, enhancing its ability to establish and spread within or beyond their progenitor populations (Levin 2012). Despite the dry nature of the climate found at Restinga de Massambaba, there is a mosaic of vegetation physiognomies growing in different environmental conditions, which dramatically increases species diversity found in the region, with estimated 664 plant species (Araujo 1992; Araujo et al. 2009). Thus, the heterogeneous nature of coastal habitats found in the region may also provide the ecological opportunity required for the persistence of the hybrid zone (Harrison 1990; Vines et al. 2003).

According to Ren et al. (2014), hybrid zones between rewarding and food-deceptive species may provide the opportunity to test how the pollinator reacts to the phenotypic admixture of floral traits selected for contrasting pollination strategies. Indeed, hybrid flowers attract both diurnal and nocturnal visitors, suggesting an admixture of floral signals, potentially combining shape, color and scent, and future studies should address this topic in more details. Considering that hybrid fertility may persist for millions of years (Levin 2012), the window of opportunity for speciation in this hybrid zone is still potentially open.

Conclusions

Overall, our results indicate strong but permeable barriers to gene exchange between *E. denticulatum* and *E. orchidiflorum*, preventing species collapse in this hybrid zone. Both prezygotic (habitat isolation) and postzygotic (hybrid sterility) mechanisms made a substantial contribution to total isolation. Although the low levels of hybrid fertility and absence of ecological segregation supports a typical tension zone model (balance between dispersal and selection against hybrids), the question remains as to what extent ecological selection associated with the habitat 'mosaic' of the coastal vegetation accounts for the persistence of the hybrid zone (Barton and Hewitt 1985; Vines et al. 2003). This question may be addressed by studying spatial patterns of hybridization, or associations between hybrid genomic composition and environmental variables (Vallejo-Marín and Hiscock 2016). Having genomic data (e.g. SNPs distributed across the genome) may also allow us to classify hybrid plants into different hybrid classes, which may be associated to different levels of nectar production employing the method recently proposed by Scopece et al. (2020). This may allow to gain clues on the genetic architecture of this important trait giving insights on the frequent transitions between deceptive and rewarding pollination strategies in orchids (Cardoso-Gustavson et al. 2018).

Acknowledgements We thank Karolyne Wanessa de Jesus, Cláudio Nicoletti Fraga, Melissa Bocayuva and Sylvio Rodrigues Pereira for the assistance in sampling plants at Restinga de Massambaba. The authors thank Espaço da Escrita – Pró-Reitoria de Pesquisa - UNICAMP - for the language services provided.

Authors' contributions FP and GS initiated and designed the study. BLA collected data on reproductive biology. RMM, APM and EFM performed cytogenetic analysis. All authors contributed to the writing and all of them read and approved the final manuscript.

Funding Funding for this study was provided by fellowships from CNPq – PIBIC to BLA and FAEPEX (FUNCAMP) to FP. This study was also financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

Compliance with ethical standards

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

References

Abbott RJ, Brennan AC (2014) Altitudinal gradients, plant hybrid zones and evolutionary novelty. Philos Trans R Soc Lond B Biol Sci 369:20130346

Almeida AM, Figueiredo RA (2003) Ants visit nectaries of *Epidendrum denticulatum* (Orchidaceae) in a Brazilian rainforest: effects on herbivory and pollination. Braz J Biol 63:551–558

Araujo DD (1992) Vegetation types of sandy coastal plains of tropical Brazil: a first approximation. In: Seeliger U (ed) Coastal plant communities of Latin America. Academic Press, San Diego, pp 337–347

Araujo DSD, de Sá CFC, Fontella-Pereira J, Garcia DS, Ferreira MV, Paixão RJ, Fonseca-Kruel VS et al (2009) Área de Proteção Ambiental de Massambaba, Rio de Janeiro: caracterização fitofisionômica e florística. Rodriguésia 60:67–96

Armbruster WS, Muchhala N (2009) Associations between floral specialization and species diversity: cause, effect, or correlation? Evol Ecol 23:159–179

Baack E, Melo MC, Rieseberg LH, Ortiz-Barrientos D (2015) The origins of reproductive isolation in plants. New Phytol 207:968–984

Barton NH, Hewitt GM (1985) Analysis of hybrid zones. Ann Rev Ecol Syst 16:113–148

Bolnick DI, Near TJ (2005) Tempo of hybrid inviability in centrarchid fishes (Teleostei: Centrarchidae). Evolution 59:1754–1767

Braga PIS (1977) Aspectos biológicos das Orchidaceae de uma campina da Amazônia Central. Acta Amazon 7:5–89

Burton TL, Husband B (2000) Fitness differences among diploids, tetraploids, and their triploid progeny in *Chamerion angustifolium*: mechanisms of inviability and implications for polyploid evolution. Evolution 54:1182–1191

Cahenzli F, Bonetti C, Erhardt A (2018) Divergent strategies in pre-and postzygotic reproductive isolation between two closely related *Dianthus* species. Evolution 72:1851–1862

Cardoso-Gustavson P, Saka MN, Pessoa EM, Palma-Silva C, Pinheiro F (2018) Unidirectional transitions in nectar gain and loss suggest food deception is a stable evolutionary strategy in *Epidendrum* (Orchidaceae): insights from anatomical and molecular evidence. BMC Plant Biol 18:1–13

Chapman MA, Abbott RJ (2010) Introgression of fitness genes across a ploidy barrier. New Phytol 186:63–71

Chase MW, Christenhusz MJM, Fay MF, Byng JW, Judd WS, Soltis DE et al (2016) An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG IV. Bot J Linn Soc 181:1–20

Colmer TD, Voesenek LCJ (2009) Flooding tolerance: suites of plant traits in variable environments. Funct Plant Biol 36:665–681

Coyne JA, Orr HA (2004) Speciation. Sinauer Associates, Sunderland

Cozzolino S, Scopece G (2008) Specificity in pollination and consequences for postmating reproductive isolation in deceptive Mediterranean orchids. Philos Trans R Soc Lond B Biol Sci 363:3037–3046

Cozzolino S, D'Emerico S, Widmer A (2004) Evidence for reproductive isolate *selection* in Mediterranean orchids: karyotype differences compensate for the lack of pollinator specificity. Proc R Soc Lond B Biol Sci 271:S259–S262

Cozzolino S, Schiestl FP, Müller A, De Castro O, Nardella AM, Widmer A (2005) Evidence for pollinator sharing in Mediterranean nectar-mimic orchids: absence of premating barriers? Proc R Soc Lond B Biol Sci 272:1271–1278

Doležel J, Greilhuber J, Suda J (2007) Estimation of nuclear DNA content in plants using flow cytometry. Nat Protoc 2:2233–2244

Grant V (1981) Plant speciation. Columbia University Press, New York

Hágsater E, Soto Arenas MA (2005) *Epidendrum* L. In: Pridgeon AM, Cribb PJ, Chase MW, Rasmussen FN (eds) Genera Orchidacearum 4. Oxford University Press, Oxford, pp 236–251
Harrison RG (1990) Hybrid zones: windows on evolutionary process. Oxf Surv Evol Biol 7:69–128
Hewitt G (2000) The genetic legacy of the Quaternary ice ages. Nature 405:907–913
Hipperson H, Dunning LT, Baker WJ, Butlin RK, Hutton I, Papadopulos AST, Savolainen V et al (2016) Ecological speciation in sympatric palms: 2. Pre-and post-zygotic isolation. J Evol Biol 29:2143–2156
Jersáková J, Johnson SD, Kindlmann P (2006) Mechanisms and evolution of deceptive pollination in orchids. Biol Rev 81:219–235
Kentner EK, Mesler MR (2000) Evidence for natural selection in a fern hybrid zone. Am J Bot 87:1168–1174
Köhler C, Scheid OM, Erilova A (2010) The impact of the triploid block on the origin and evolution of polyploid plants. Trends Genet 26:142–148
Levin DA (2012) The long wait for hybrid sterility in flowering plants. New Phytol 196:666–670
Lexer C, Welch ME, Raymond O, Rieseberg LH (2003) The origin of ecological divergence in *Helianthus paradoxus* (Asteraceae): selection on transgressive characters in a novel hybrid habitat. Evolution 57:1989–2000
Lowry DB, Modliszewski JL, Wright KM, Wu CA, Willis JH (2008) The strength and genetic basis of reproductive isolating barriers in flowering plants. Philos Trans R Soc Lond B Biol Sci 363:3009–3021
Mallet J (2007) Hybrid speciation. Nature 446:279–283
Marques I, Draper D, Riofrío L, Naranjo (2014) Multiple hybridization events, polyploidy and low postmating isolation entangle the evolution of neotropical species of *Epidendrum* (Orchidaceae). BMC Evol Biol 14:1–14
Marques I, Jürgens A, Aguilar JF, Feliner GN (2016) Convergent recruitment of new pollinators is triggered by independent hybridization events in *Narcissus*. New Phytol 210:731–742
Marques I, Loureiro J, Draper D, Castro M, Castro S (2018) How much do we know about the frequency of hybridisation and polyploidy in the Mediterranean region? Plant Biol 20:21–37
Martin NH, Willis JH (2007) Ecological divergence associated with mating system causes nearly complete reproductive isolation between sympatric *Mimulus* species. Evolution 61:68–82
Martin NH, Bouck AC, Arnold ML (2006) Detecting adaptive trait introgression between *Iris fulva* and *I. brevicaulis* in highly selective field conditions. Genetics 172:2481–2489
Moraes AP, Chinaglia M, Palma-Silva C, Pinheiro F (2013) Interploidy hybridization in sympatric zones: the formation of *Epidendrum fulgens* × *E. puniceoluteum* hybrids (Epidendroideae, Orchidaceae). Ecol Evol 3:3824–3837
Mota MR, Pinheiro F, Leal BSS, Wendt T, Palma-Silva C (2019) The role of hybridization and introgression in maintaining species integrity and cohesion in naturally isolated inselberg bromeliad populations. Plant Biol 21:122–132
Natalis LC, Wesselingh RA (2012) Shared pollinators and pollen transfer dynamics in two hybridizing species, *Rhinanthus minor* and *R. angustifolius*. Oecologia 170:709–721
Nosil P, Vines TH, Funk DJ (2005) Reproductive isolation caused by natural selection against immigrants from divergent habitats. Evolution 59:705–719
Orr HA, Turelli M (2001) The evolution of postzygotic isolation: accumulating Dobzhansky–Muller incompatibilities. Evolution 55:1085–1094
Pachon AMR (2016) Filogeografia a partir de DNA de cloroplasto da orquídea neotropical *Epidendrum orchidiflorum* (Orchidaceae: Laeliinae) no Brasil. Ph.D. Dissertation, Universidade de São Paulo, Brazil
Paudel BR, Burd M, Shrestha M, Dyer AG, Li QJ (2018) Reproductive isolation in alpine gingers: how do coexisting *Roscoea* (*R. purpurea* and *R. tumjensis*) conserve species integrity? Evolution 72:1840–1850
Pegoraro L, Cafasso D, Rinaldi R, Cozzolino S, Scopece G (2016) Habitat preference and flowering-time variation contribute to reproductive isolation between diploid and autotetraploid *Anacamptis pyramidalis*. J Evol Biol 29:2070–2082
Pinheiro F, Cozzolino S (2013) *Epidendrum* (Orchidaceae) as a model system for ecological and evolutionary studies in the Neotropics. Taxon 62:77–88
Pinheiro F, de Barros F, Palma-Silva C, Meyer D, Fay MF, Suzuki RM, Lexer C, Cozzolino S (2010) Hybridization and introgression across different ploidy levels in the Neotropical orchids *Epidendrum fulgens* and *E. puniceoluteum* (Orchidaceae). Mol Ecol 19:3981–3994
Pinheiro F, Cardoso-Gustavson P, Suzuki RM, Abrao MCR, Guimaraes LR, Draper D, Moraes AP (2015) Strong postzygotic isolation prevents introgression between two hybridizing Neotropical orchids, *Epidendrum denticulatum* and *E. fulgens*. Evol Ecol 29:229–248

Pinheiro F, de Melo e Gouveia TMZ, Cozzolino S, Cafasso D, Cardoso-Gustavson P, Suzuki RM, Palma-Silva C (2016) Strong but permeable barriers to gene exchange between sister species of *Epidendrum*. Am J Bot 103:1472–1482

Ren MX, Cafasso D, Lumaga MRB, Cozzolino S (2014) Low pollination success of hybrids between nectar-rewarding and food-deceptive orchids. Plant Syst Evol 300:1985–1993

Rieseberg LH, Willis JH (2007) Plant speciation. Science 317:910–914

Rodrigues BJ (1877) Genera et species Orchidearum Novarum v. 1. Typographia Nacional, Sebastianópolis

São Leão LC (2012) Estudos reprodutivos em duas espécies simpátricas de Epidendrum L. (Epidendroideae–Orchidaceae) em vegetação de restinga. M.Sc. Thesis. Universidade Federal do Rio de Janeiro, Brazil

São Leão LC, Sá-Haiad B, Araujo Rodarte AT, Pimentel RR, Benevides CR, Santiago-Fernandes LDR, Lima HA (2019) Reproductive biology of two synchronopatric neotropical species of *Epidendrum* (Orchidaceae). Flora 251:95–104

Sasa MM, Chippindale PT, Johnson NA (1998) Patterns of postzygotic isolation in frogs. Evolution 52:1811–1820

Schemske DW (2010) Adaptation and the origin of species. Am Nat 176:S4–S25

Scopece G, Musacchio A, Widmer A, Cozzolino S (2007) Patterns of reproductive isolation in Mediterranean deceptive orchids. Evolution 61:2623–2642

Scopece G, Widmer A, Cozzolino S (2008) Evolution of postzygotic reproductive isolation in a guild of deceptive orchids. Am Nat 171:315–326

Scopece G, Cozzolino S, Johnson SD, Schiestl FP (2010) Pollination efficiency and the evolution of specialized deceptive pollination systems. Am Nat 175:98–105

Scopece G, Croce A, Lexer C, Cozzolino S (2013) Components of reproductive isolation between *Orchis mascula* and *Orchis pauciflora*. Evolution 67:2083–2093

Scopece G, Palma-Silva C, Cafasso D, Lexer C, Cozzolino S (2020) Phenotypic expression of floral traits in hybrid zones provides insights into their genetic architecture. New Phytol 227:967–975

Serrato-Capuchina A, Matute DR (2018) The role of transposable elements in speciation. Genes 9:254

Shang H, Hess J, Pickup M, Field DL, Ingvarsson PK, Liu J, Lexer C (2020) Evolution of strong reproductive isolation in plants: broad-scale patterns and lessons from a perennial model group. Philos Trans R Soc Lond B Biol Sci 375:20190544

Sobel JM, Chen GF (2014) Unification of methods for estimating the strength of reproductive isolation. Evolution 68:1511–1522

Todesco M, Pascual MA, Owens GL, Ostevik KL, Moyers BT, Hübner S, Rieseberg LH et al (2016) Hybridization and extinction. Evol Appl 9:892–908

Tremblay RL, Ackerman JD, Zimmerman JK, Calvo RN (2005) Variation in sexual reproduction in orchids and its evolutionary consequences: a spasmodic journey to diversification. Biol J Linn Soc 84:1–54

Turissini DA, McGirr JA, Patel SS, David JR, Matute DR (2018) The rate of evolution of postmating-prezygotic reproductive isolation in *Drosophila*. Mol Biol Evol 35:312–334

Valladares F, Niinemets Ü (2008) Shade tolerance, a key plant feature of complex nature and consequences. Ann Rev Ecol Evol Syst 39:237–257

Vallejo-Marín M, Hiscock SJ (2016) Hybridization and hybrid speciation under global change. New Phytol 211:1170–1187

Vallejo-Marín M, Cooley AM, Lee MY, Folmer M, McKain MR, Puzey JR (2016) Strongly asymmetric hybridization barriers shape the origin of a new polyploid species and its hybrid ancestor. Am J Bot 103:1272–1288

Vega Y, Marques I, Castro S, Loureiro J (2013) Outcomes of extensive hybridization and introgression in *Epidendrum* (Orchidaceae): can we rely on species boundaries? PLoS ONE 8:e80662

Vines TH, Kohler SC, Thiel A, Ghira I, Sands TR, MacCallum CJ, Barton NH, Nurnberger B (2003) The maintenance of reproductive isolation in a mosaic hybrid zone between the fire-bellied toads *Bombina bombina* and *B. variegata*. Evolution 57:1876–1888

Xu S, Schlüter PM, Scopece G, Breitkopf H, Gross K, Cozzolino S, Schiestl FP (2011) Floral isolation is the main reproductive barrier among closely related sexually deceptive orchids. Evolution 65:2606–2620

Zitari A, Scopece G, Helal AN, Widmer A, Cozzolino S (2012) Is floral divergence sufficient to maintain species boundaries upon secondary contact in Mediterranean food-deceptive orchids? Heredity 108:219–228

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