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COMPONENTS OF REPRODUCTIVE ISOLATION BETWEEN *ORCHIS MASCULA* AND *ORCHIS PAUCIFLORA*

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Studies of the strength and nature of reproductive isolation (RI) between species can greatly contribute to our understanding of speciation. Although the role of RI in speciation is well recognized, there is a dearth of information on the contributions of different barriers between related plant species. Here, we estimated multiple components of RI between two Mediterranean orchid sister species (*Orchis mascula* and *Orchis pauciflora*), disentangling the strength and absolute contributions of seven different isolating mechanisms. Our survey includes one prepollination, two postpollination prezygotic (pollen–stigma incompatibility, conspecific pollen precedence), two intrinsic postzygotic (embryo mortality and hybrid sterility) and two extrinsic postzygotic (hybrid habitat differentiation and hybrid pollination) isolating mechanisms. We found strong RI between the investigated species, although none of the barriers were able to completely impede gene flow. Five isolating mechanisms contributed positively to the maintenance of species boundaries. Contrary to most surveys of isolating mechanisms, our data speak against a clear predominance of prepollination or of prezygotic barriers but confirm the emerging pattern of multiple barriers contributing to the maintenance of species integrity. These findings suggest an allopatric condition during early phases of species divergence. We discuss our data in the wider context of previous studies carried out in this orchid group by using a comparative approach.

KEY WORDS: **Postpollination isolation, postzygotic isolation, prepollination isolation, prezygotic isolation, speciation.**

The actual diversity of living beings is a direct consequence of the reproductive barriers that allowed species' gene pools to differentiate and evolve independently. Many different isolating mechanisms have been described and characterized among sexually reproducing organisms and their detection among closely related taxa is highly informative for the understanding of the speciation process (Coyne and Orr 2004). Among flowering plants, reproductive isolation (RI) can be achieved through the action of pre- and postpollination mechanisms (Grant 1971). Postpollination barriers can act either before or after the fusion of parental gametes and are consequently categorized as pre- and postzygotic. The latter are often separated into intrinsic (as embryo mortality, hybrid inviability, and sterility) and extrinsic (as ecological and behavioral sterility) barriers (Coyne and Orr 2004). In general, a complete understanding of speciation among sexual organisms requires an understanding of the accumulation of these reproductive isolating barriers among lineages that are still in the process of diverging or are recently diverged (Scopece et al. 2010).

Overall, two alternative approaches have been used to unravel speciation scenarios through the estimation of RI, namely a comparative and a case study approach (Dopman et al. 2010). These approaches, even if imperfect, have contributed greatly to a better understanding of speciation and, through the detection and classification of the type and relevance of barriers that contribute

to RI, have had the merit of encouraging studies on the genetic basis of speciation.

The comparative approach takes origin from the influential study of Coyne and Orr (1989) on *Drosophila* and is based on the measurement of the strength of reproductive isolating mechanisms across large groups of taxa that vary in divergence time and on their comparison with genetic distances as a proxy of divergence time. A number of studies have investigated patterns of RI using the comparative approach in animals (Coyne and Orr 1989, 1997; Sasa et al. 1998; Presgraves 2002; Malone and Fontenot 2008) and plants (Moyle et al. 2004; Archibald et al. 2005; Scopece et al. 2007, 2008; Jewell et al. 2012). These studies have shed light on the evolutionary rates of different types of isolating mechanisms and have shown that prezygotic mechanisms generally evolve erratically and rapidly whereas the evolution of postzygotic mechanisms appears to be more gradual and clock-like.

Although the comparative approach contributed greatly to our understanding of speciation, it suffers from a major shortcoming, because it generally takes into account only a single or a few individual components of RI, although it is increasingly clear that the maintenance of species integrity is the result of the combination of a variety of mechanisms (Lowry et al. 2008) and that the complementary evolution of multiple types of reproductive barriers plays a key role in speciation (Coyne and Orr 2004; Matsubayashi and Katakura 2009). Indeed, even very closely related species, despite the small time elapsed since divergence, can be (and typically are) separated by multiple barriers that act together in preventing gene exchange (Ramsey et al. 2003; Lowry et al. 2008). That is, although individual barriers often remain incomplete until long after speciation (Gourbiere and Mallet 2010), when taking into account the joint action of multiple components, total RI appears to be generally strong (Lowry et al. 2008) suggesting that the patterns of RI separating the species cannot be fully understood without taking into account multiple components. As a consequence, one goal of speciation biology has become the fine-scale determination of the strength and relative importance of multiple components of RI in case study species pairs (Ramsey et al. 2003; Nosil et al. 2005; Martin and Willis 2007; Dopman et al. 2010). Although the lack of independent contrasts precludes proper tests of the temporal order of evolution of individual barriers (Coyne and Orr 1997), the detection of multiple components of RI in single case study can contribute to different areas of the speciation debate. Following this approach it is possible to characterize the architecture of RI, that is, if RI is maintained by the joint action of multiple isolating mechanisms or if there are single major isolating mechanisms responsible of the isolation (Lowry et al. 2008). This outcome is of particular relevance because these two alternative scenarios can have different effects on the timing and biogeographic condition of speciation. Furthermore, quantifying different components of isolation can help in understanding the relative contribution of pre- versus postpollination or pre- versus postzygotic mechanisms, and to estimate total RI and the asymmetry of reproductive barriers.

So far, only a handful of studies have quantified the strength of individual reproductive isolating mechanisms among closely related plant species (reviewed in Lowry et al. 2008). These studies have been conducted on closely related species of *Conospermum* (Morrison et al. 1994), *Penstemon* (Chari and Wilson 2001), *Mimulus* (Ramsey et al. 2003; Martin and Willis 2007), *Chamerion* (Husband and Sabara 2004), *Costus* (Kay 2006), *Jamesbrittenia* (Hoffmann et al. 2008), and *Petunia* (Dell'Olivo et al. 2011). Overall, these studies support the general claims that multiple reproductive isolating mechanisms contribute to total isolation and that prezygotic barriers make a greater contribution than postzygotic ones (reviewed in Lowry et al. 2008). However, to draw a general picture of patterns of RI between closely related plant species, more case studies are needed spanning across different groups and covering the maximum number of potential barriers.

Here, we analyze the strength and absolute contribution of individual isolating barriers and estimated total RI between two orchid sister species, *Orchis mascula* and *Orchis pauciflora*. The two species belong to a group of Mediterranean food-deceptive Orchidinae in which patterns of RI have been recently investigated using the comparative approach (Scopece et al. 2007, 2008). The present research, combined with previous investigations, will thus allow to employing the two alternative approaches (comparative and case study) on a group of orchids strengthening our knowledge of isolating mechanisms in one of the most species-rich families in the plant kingdom. Our survey of isolating mechanisms includes one prepollination, two postpollination prezygotic (pollen–stigma incompatibility, conspecific pollen precedence), two intrinsic postzygotic (embryo mortality and hybrid sterility), and two extrinsic postzygotic (hybrid habitat differentiation and hybrid pollination) isolating mechanisms comprising a total of seven stages. Using these data, we aim to answer the following questions:

i. Are *O. mascula* and *O. pauciflora* separated primarily by a single barrier or by the joint action of multiple components of RI?
ii. What is the strength and absolute contribution of each individual component, what are those of prepollination versus postpollination and of prezygotic versus postzygotic isolating mechanisms to total RI?
iii. What does the architecture of RI tell us about the mechanisms of this speciation event?
iv. What are the relative merits of comparative and case study approaches?

Materials and Methods

STUDY SYSTEM

O. mascula and *O. pauciflora* are sister species in the subfamily Orchidinae (Aceto et al. 1999; Bateman et al. 2003). *O. mascula* is a widespread European species that inhabits sunny meadows or calcareous grasslands from Sweden to the southern Mediterranean countries (Sundermann 1980); *O. pauciflora* has a more strictly Mediterranean distribution range, and inhabits rocky calcareous soils in the southeastern and central part of the Mediterranean area. In Italy, these distributions overlap at intermediate altitude on the Apennine mountain chain where the two species form hybrid zones (Pellegrino et al. 2000; Cozzolino et al. 2006). The two species have similar flower morphology, but show a strong difference in color (*O. mascula* is purple-red flowered and *O. pauciflora* is pale-yellow flowered), inflorescence length (20–60 cm in *O. mascula*, 10–30 cm in *O. pauciflora*; Delforge 2005), and number of flowers (15–50 in *O. mascula*, 2–8 in *O. pauciflora*; Delforge 2005). Both species have long spurs but do not produce nectar, and rely on a nonmodel generalized food-deceptive strategy for their pollination (Van der Cingel 1995). *O. mascula* and *O. pauciflora* are nonautogamous, self-compatible and have overlapping phenologies (flowering in April–May). Hymenopterans are the more common pollinators of *O. mascula* (especially *Bombus* sp., but also *Psithirus*, *Eucera*, *Andrena*, *Osmia*, *Anthophora*; Nilsson 1983), and *O. pauciflora* (especially *Bombus* queens; Valterovà et al. 2007).

MANUAL CROSSES AND REPRODUCTIVE SUCCESS ESTIMATES

Hand pollination experiments were performed as described in Scopece et al. (2007). All individuals used in the experiments were collected from natural populations in the Cilento e Vallo di Diano National Park (Southern Italy). We performed intra- and interspecific pollinations on *O. mascula* and *O. pauciflora* individuals. Then to test hybrid performances, we performed bidirectional crosses among hybrid individuals and both parental species. To test conspecific pollen precedence, on both species, we performed mixed pollinations with an equal amount of intra- and interspecific pollen at different time intervals.

To estimate pollination success, we marked individuals during flowering time (to ensure the correct assignment of individuals for each taxon) and counted the number of flowers of each labeled individual. After roughly 1 month, we checked all the marked individuals for the presence of fruits. For each individual, pollination success was then calculated as the number fruits produced relative to the number of flowers in the inflorescence.

SEED POOL PATERNITY TEST

Intra- and interspecifically pollinated fruits were detected by the analysis of single nucleotide polymorphisms (SNPs). This analysis was performed by aligning ITS1 sequences of *O. mascula* and *O. pauciflora* (GenBank accessions: Z940881 and Z940991) and searching for potential polymorphisms. A G / T substitution (G in *O. mascula* and T in *O. pauciflora*) was selected flanked by sequences that allowed designing a specific Snapshot primer (5′-CGCACACCCATCCATTCGCTGCATAAGAACC-3′). DNA from pooled seeds from a single fruit was extracted following Doyle and Doyle (1987). Seed pool genotyping was then performed by single-base extension sequencing using the Snapshot kit (Applied Biosystems, Foster City, CA). Polymerase chain reaction (PCR) amplification of ITS1 was performed using primers and conditions described in Aceto et al. (1999) and followed by exonuclease I and SAP treatments (Fermentas Inc., Hannover, MD). Then 1 μL of purified PCR product, 1 μL 5× PCR buffer, 1 μL Snapshot kit mix, 0.2 μL of 2 M specific snapshot primer (SEQ), and 2.8 μL H_2O were mixed and subjected to 30 cycles of 95°C for 20 sec, 50°C for 1 min, and 60°C for 1 min. After PCR purification by SAP treatment was then performed by addition of 0.8 μL SAP and 0.8 μL SAP dilution buffer. Products were identified after ABI 3130 capillary electrophoresis run by using GeneMapper v3.1 (Applied Biosystems).

RI INDICES

All the RI indices were assessed basing on experimental and / or on literature data gained with the specific aim of comparing intra- and interspecific performances of *O. mascula* and *O. pauciflora*. To allow the comparison among different isolation stages, following the method initially proposed by Coyne and Orr (1989) and widely applied in subsequent studies, all the indices were calculated so that they can potentially range from 0 (no isolation) to 1 (complete isolation). Negative values represent cases in which interspecific performances were higher then intraspecific ones. Below is a description of each of the indices ordered from the early acting to the late acting ones.

Prepollination isolation index ($RI_{PRE\text{-}POLL}$).

To be comparable and mathematically equivalent with postpollination isolation indices, the calculation of indices accounting for prepollination isolation requires the exact estimation of relative abundances of available mates (Martin and Willis 2007). This would allow us to apply the formula: RI = 1 − (observed / expected interspecific mating) / (observed / expected intraspecific mating), where the expected values are calculated basing on the different abundance of potential mates (Martin and Willis 2007). However, the precise quantification of the number of potential mates in field conditions is an extremely difficult task because it basically requires the collection of data for each isolation index using an experimental setup in which all parameters are controlled for (as for instance, phenology, number of flowers,

number of pollen grains, distance among individuals; e.g., Xu et al. 2011). To circumvent these problems, we calculated a synthetic index that accounts for all the potential isolating mechanisms that occur before the arrival of interspecific pollen on the stigma of each of the investigated species. Among these mechanisms, there are differences in flower phenology, in the abundance of the parental species and, more importantly, all factors related to floral isolation. Including all these prepollination mechanisms together within one synthetic index makes use of the assumption that, in the absence of any of these barriers, we can expect an equal probability of intra- and interspecific matings in sympatric populations (i.e., 50% each). This allows us to apply a simplified version of the formula of Martin and Willis (2007) as RIS $= 1 -$ (observed interspecific mating) / (observed intraspecific mating).

To calculate this prepollination index despite the known difficulty of directly observing pollination in deceptive species, we used an indirect approach to quantify the number of interspecific pollinations in sympatric populations of *O. mascula* and *O. pauciflora*. That is, after categorizing all the individuals and labeling them, we randomly collected ripe fruits from a sympatric zone in the Cilento e Vallo di Diano National Park and performed an analysis of SNPs (as described earlier) to assess the number of hybrid fruits (showing two peaks in the SNPs analysis; i.e., produced after interspecific pollinations) and the number of pure parental fruits (showing a single peak in the SNPs analysis; i.e., produced after intraspecific pollinations). For each species, the prepollination isolation index ($RI_{\text{PRE-POLL}}$) was then calculated as: $RI_{\text{PRE-POLL}} = 1 -$ (number of hybrid fruits / number of pure parental fruits).

Because from our crossing data we found that a proportion of interspecific pollinations failed to trigger fruit formation, to discount this proportion from our estimation, we corrected the estimation of $RI_{\text{PRE-POLL}}$ by adding to the observed number of hybrid fruits the expected number of fruits that would have formed if all the interspecific pollinations were able to produce fruits. Even if $RI_{\text{PRE-POLL}}$ was calculated basing on molecular characterization of the seed pool (i.e., after that pollination and embryo development occurred), this index is not influenced by other postpollination barriers that precede viable seed formation. Indeed, our crossing data show that all interspecific pollinations that trigger fruit formation always produce a proportion of viable embryos (i.e., thus detectable using a qualitative SNPs analysis). Furthermore, the case of contemporary intra- and interspecific pollination of the same flower, that could be undetected using this approach, is extremely rare in deceptive orchids because flowers of these species are scantily visited and have a rapid withering after pollination (Faegri and Van der Pijl 1979).

Pollen–stigma incompatibility isolation index ($RI_{\text{POLL\_STIGMA}}$)

In orchids, female gametophyte development and consequent ovary enlargement (i.e., fruit formation) follows the arrival of pollen on the stigma and precedes the formation of the zygote (Zhang and O'Neill 1993). Therefore, the hand pollinations allowed distinguishing a postpollination prezygotic isolation stage based on the comparison between fruit development after intra- or interspecific pollinations. This index was calculated as: $RI_{\text{POLL\_STIGMA}} = 1 -$ (% fruit formed in interspecific crosses / % fruit formed in intraspecific crosses; cf. Scopece et al. 2007).

Conspecific pollen precedence isolation index (RI_{CPP})

In plants, sessility and the lack of mate choice and courtship behavior imply the frequent presence of intra- and interspecific pollen on stigmas and in many species pairs, mechanisms of conspecific pollen precedence have been described (Howard 1999). To verify the existence of mechanisms that favor intraspecific pollen in mixed pollinations between the two investigated species, we performed, on both species mixed pollination treatments (i.e., with an equal amount of inter- and intraspecific pollen) at two different time intervals, that is by contemporarily depositing inter- and intraspecific pollen and by depositing intraspecific pollen 12 h after the interspecific pollen. In contemporary pollinations, the existence of conspecific pollen precedence should produce a decrease in the number of hybrid fruits with respect to the crosses in which intraspecific pollen was deposited after 12 h. Therefore, the index of conspecific pollen precedence was calculated as: $RI_{\text{CPP}} = 1 -$ (% pure parental fruits after 12 h / % pure parental fruits in contemporary pollinations).

Embryo mortality isolation index (RI_{EMBRYO})

An early postzygotic isolation index was calculated using seed viability data after inter- and intraspecific pollinations. RI_{EMBRYO} was calculated as the proportion of viable seeds obtained in interspecific pollinations, relative to the proportion of viable seeds in intraspecific pollinations within each parental species: $RI_{\text{EMBRYO}} = 1 -$ (% viable seeds in interspecific crosses / % viable seeds in intraspecific crosses; Scopece et al. 2007).

Hybrid sterility isolation index (RI_{HS})

The hybrid sterility index was estimated using crossing data. The experimental design with bidirectional backcrosses between the parental species and the hybrids allowed estimating two components of hybrid sterility, a male and a female component. Because in this study we are interested in reproductive barriers between *O. mascula* and *O. pauciflora* we did not take into account crossing combinations involving only hybrid individuals that do not influence levels of gene flow between parentals.

For all the crossing directions, hybrid sterility is composed of two stages, fruit production and viable seed production. Hybrid sterility indices were calculated separately for each stage. For each species, the fruit production component was defined as: $F = 1 -$ (number of fruits produced / number of pollinated flowers). Similarly, the seed production component was estimated using the formula: $S = 1 -$ (viable seeds / total number of counted seeds). As previously done in Scopece et al. (2008), we calculated the linear sequential combination of F and S for all the crossing directions ($F\_S = F + (1 - F) \times S$). We performed these calculations independently for crosses in which hybrid individuals were the pollen donors and for crosses in which hybrid individuals were the seed parent, to gain the male ($RI_{HS}M$) and female components ($RI_{HS}F$) of hybrid sterility, respectively. The mean of the two combined values represents our hybrid sterility index (RI_{HS}).

Hybrid habitat differentiation isolation index ($RI_{HABDIFF}$)

Niche differentiation between parentals and their hybrid is an important isolating mechanism. Indeed, if natural selection removes hybrids from the sympatric zones, this would decrease the opportunity of backcrosses and thus of hybrid functioning as a bridge between the parental genomes. To explore this extrinsic postzygotic mechanism, we used distribution data from Nazzaro et al. (1992) and estimated the number of 4 km^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). $RI_{HABDIFF}$ was calculated as: 1 – (number of heterospecific quadrats / number of heterospecific quadrats + number of semi-heterospecific quadrats + number of conspecific quadrats).

Hybrid pollination isolation index (RI_{HPOLL})

In plants it has often been reported that gene flow between closely related species may be impeded by the breakdown of phenotypic floral traits involved in attraction of pollinators (Schluter 2000). To take into account this potential mechanism, we calculated an extrinsic postzygotic isolation index describing the hybrid capability of attracting pollinators. To do this, we compared natural pollination success (number of fruits produced relative to the total number of flowers in an inflorescence) of hybrid versus parental individuals. Hybrid pollination isolation index was then calculated as: $RI_{HPOLL} = 1 -$ (hybrid fruit set/parental fruit set). Because from our controlled hand pollinations we found that a proportion (10.1%) of pollinations on hybrid individuals fail to trigger fruit formation, we inserted a correction to distinguish the effect of pollinator preference from that of intrinsic hybrid sterility. To discount this proportion, we thus corrected the estimation of RI_{HPOLL} by adding to the observed number of fruits in hybrid individuals, the expected number of fruits that would have formed if all the pollination events were able to produce fruits.

TOTAL RI

We estimated total RI (RI_{TOT}) between *O. mascula* and *O. pauciflora* following the methods proposed by Coyne and Orr (1989, 1997) and modified by Ramsey et al. (2003), as the product of individual isolating mechanisms that act sequentially to prevent or limit gene flow. The strength of each isolating mechanism was calculated independently, and the absolute contribution of that mechanism (AC) was estimated as the proportional reduction in gene flow that has not been eliminated by previous stages.

To compare the contribution of prepollination versus postpollination and of prezygotic versus postzygotic mechanisms, following Lowry et al. (2008), we calculated indices of total isolation for each barrier category (i.e., prepollination: RI_{PREP_TOT}; postpollination: RI_{POSTP_TOT}; prezygotic: RI_{PREZ_TOT}; postzygotic: RI_{POSTZ_TOT}).

We considered asymmetric those isolating mechanisms for which the absolute value of differences in strength in the two possible directions was higher than 0.25.

Results

Results for all the isolation indices and for their absolute contributions to total RI are listed in Table 1.

Prepollination isolation index ($RI_{PRE\text{-}POLL}$)

From the sympatric zones, we collected a total of 113 ripe fruits. The analysis of SNPs revealed that eight out of the 80 fruits produced by *O. mascula* and seven out of the 33 fruits produced by *O. pauciflora* were produced after interspecific pollinations (i.e., showed a double peak). Because we found no differences between fruit formation after intra- and interspecific crosses for *O. mascula*, no correction was needed for this species; differently, for *O. pauciflora* intraspecific crosses triggered percentage of fruit formation higher than interspecific ones. Therefore, we corrected the observed value of hybrid fruits found on *O. pauciflora* accounting for the difference between intra- and interspecific performances in controlled hand pollinations. Consequently, $RI_{PRE\text{-}POLL}$ was 0.89 for *O. mascula* and 0.67 for *O. pauciflora*.

Pollen–stigma incompatibility isolation index ($RI_{POLL\text{-}STIGMA}$)

To estimate pollen–stigma incompatibility, we carried out a total of 145 hand pollinations (80 intraspecific and 65 interspecific). All inter- and intraspecific pollinations carried out on *O. mascula* led to fruit formation ($RI_{POLL\text{-}STIGMA} = 0.00$). On *O. pauciflora*, interspecific pollinations showed a decrease of fruit

Table 1. Strength (Str) and absolute contribution (AC) of the seven investigated reproductive isolating mechanisms.

	Prezygotic		Postpollination				Intrinsic postzygotic				Extrinsic postzygotic				
	Prepollination $RI_{PRE\text{-}POLL}$		Pollen–stigma incompatibility RI_{POLL_STIGMA}		Conspecific pollen precedence RI_{CPP}		Embryo mortality RI_{EMBRYO}		Hybrid sterility RI_{HS}		Hybrid habitat different $RI_{HABDIFF}$		Hybrid attractiveness RI_{HPOLL}		Total isolation RI_{TOT}
	Str	AC	Str	AC	Str	AC	Str	AC	Str	AC	Str	AC	Str	AC	
O. mascula	0.89	0.89	0	0	−0.29	−0.03	0.20	0.03	0.44	0.05	–	–	–		0.94
O. pauciflora	0.67	0.67	0.24	0.08	0.22	0.06	0.20	0.04	0.61	0.10	–	–	–		0.95
Hybrid	–		–				–				0	0	0.10	0.01	
Total	0.78	0.78	0.12	0.03	−0,04	−0.01	0.20	0.04	0.53	0.09	0	0	0.10	0.01	0.93
Asymmetry	0.16		0.24		0.51		0.00		0.18						

formation with respect to the intraspecific ones (69.7% vs. 91.7%; $RI_{POLL\text{-}STIGMA} = 0.24$).

Conspecific pollen precedence isolation index (RI_{CPP})

When *O. mascula* was the seed parent (i.e., received the pollen) 5.56% of contemporary mixed pollinations led to the formation of pure parental fruits; pollinations carried out by applying intraspecific pollen 12 h after interspecific pollen led to the formation of 7.14% pure parental fruits. Consequently, for *O. mascula*, RI_{CPP} was –0.29 suggesting that interspecific pollen is faster than the intraspecific one.

When *O. pauciflora* was the seed parent 4.76% of contemporary mixed pollinations led to the formation of pure parental fruits; pollinations carried out by applying intraspecific pollen 12 h after interspecific pollen led to the formation of 3.70% pure parental fruits. Consequently, for *O. pauciflora* RI_{CPP} was 0.22.

Embryo mortality isolation index (RI_{EMBRYO})

In interspecific crosses, seed viability was similar when *O. mascula* was the seed parent (71.12%) and when *O. pauciflora* was the seed parent (71.24%). Intraspecific crosses held to 89.2% of viable seeds. Consequently, RI_{EMBRYO} was 0.20 both for *O. mascula* and for *O. pauciflora*.

Hybrid sterility isolation index (RI_{HS})

In backcrosses in which the hybrids were the pollen donors and *O. mascula* individuals were the seed parents, 93% of pollinated flowers developed into fruits and seeds viability was 60% ($F_{O.\ mascula} = 0.07$; $S_{O.\ mascula} = 0.40$; RI_{HS} M = 0.44). When hybrids were the seed parents, 96% of pollinated flowers developed into fruits and 60% of seeds were viable ($F_{O.\ mascula} = 0.04$; $S_{O.\ mascula} = 0.40$; RI_{HS} F = 0.43). RI_{HS} for *O. mascula* was 0.44.

In backcrosses in which the hybrids were the pollen parents and *O. pauciflora* individuals the seed parents, 86% of pollinated flowers developed into fruits and seeds viability was 34% ($F_{O.\ pauciflora} = 0.14$; $S_{O.\ pauciflora} = 0.66$; RI_{HS} M = 0.71). When hybrids were the seed parents, 84% of pollinated flowers developed into fruits and 58% of seeds were viable ($F_{O.\ pauciflora} = 0.16$; $S_{O.\ pauciflora} = 0.42$; RI_{HS} F = 0.51). RI_{HS} for *O. pauciflora* was 0.61. Overall, RI_{HS} was 0.53.

Hybrid habitat differentiation isolation index ($RI_{HABDIFF}$)

In the area of Cilento e Vallo di Diano National park for which we got the distribution information, the hybrids occurred in 36.4 km^2 quadrats and in all of them, both the parental species were also present. As a consequence, in our calculation, $RI_{HABDIFF}$ was 0.

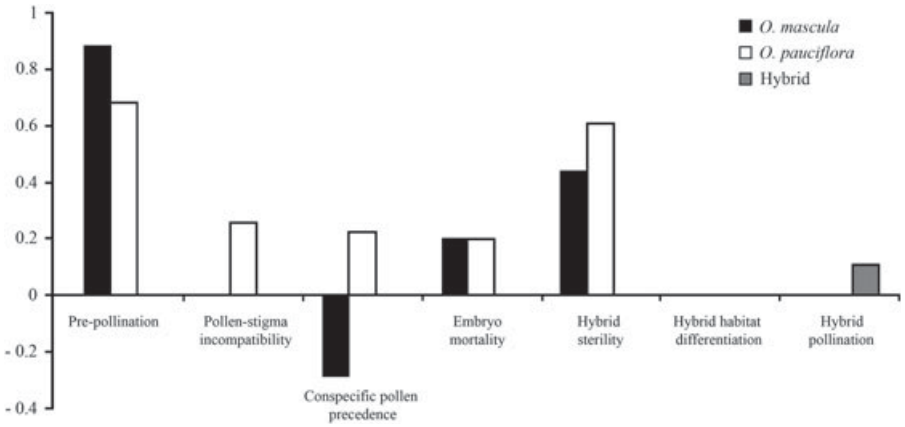

Figure 1. **Absolute strength of each investigated component of reproductive isolation in *Orchis mascula* and *Orchis pauciflora* and their hybrids.**

Hybrid pollination isolation index (RI_{HPOLL})

We estimated natural reproductive success of 151 hybrid and 1188 parental individuals (492 *O. mascula* and 696 *O. pauciflora*). Overall we found that hybrid and parental individuals showed similar levels of reproductive success. In detail, hybrid individuals produced on average 10.50 ± 1.01 fruits, whereas this value was 12.88 ± 0.49 on parental individuals (11.11 ± 0.57 on *O. mascula* and 14.14 ± 0.72 on *O. pauciflora*). Because in hand pollination experiments 10.1% of crosses in hybrid individuals failed to trigger fruit formation (6.7% when *O. mascula* and 13.5% when *O. pauciflora* were the pollen donors), we corrected the observed value of fruits found in hybrid individuals accounting for the difference with intraspecific hand pollinations. Consequently, RI_{HPOLL} was 0.10.

Total RI (RI_{TOT}) was 0.93. We did not find a clear preponderance of prepollination or postpollination mechanisms ($RI_{PREP\_TOT} = 0.78$; $RI_{POSTP\_TOT} = 0.69$; see Fig. 2). Similarly, prezygotic mechanisms were of comparable strength with postzygotic ones ($RI_{PREZ\_TOT} = 0.80$; $RI_{POSTZ\_TOT} = 0.66$; see Fig. 2).

Only one isolation index showed a difference higher than 0.25 when calculated in the two different directions (Conspecific Pollen Precedence: −0.29 in *O. mascula* and 0.22 in *O. pauciflora*, Diff. 0.51: see Fig 1).

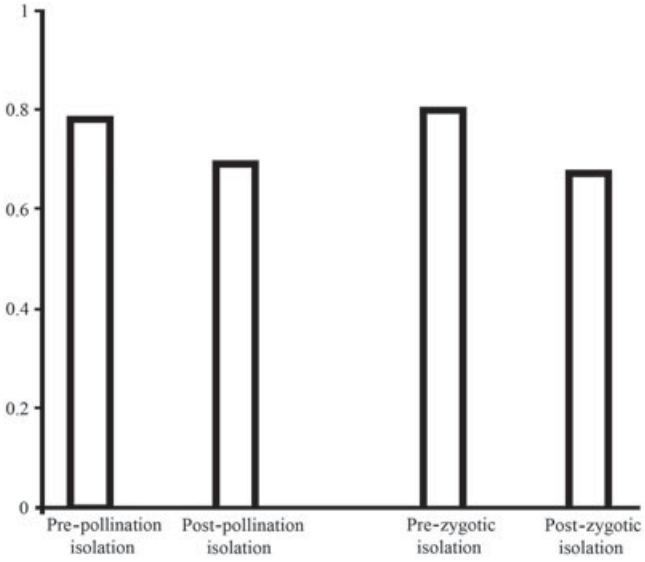

Figure 2. **Combined strength of prepollination versus postpollination and prezygotic vs. postzygotic isolating mechanisms.**

Discussion

In this study we estimated seven different mechanisms of RI between two Mediterranean orchid species, *O. mascula* and *O. pauciflora*. We also calculated total isolation following the approach proposed by Ramsey et al. (2003) as the product of individual isolating mechanisms that act sequentially. This method has been questioned by Martin and Willis (2007) who argued that the underlying assumption that successive isolating stages act independently and apply uniformly to both species is commonly violated, because premating barriers may not act uniformly in all individuals of the parental species and asymmetry in more than one barrier impedes the use of a simple multiplicative function. To take these concerns into account, we calculated a single synthetic prepollination isolation index and took note that asymmetry was limited to a single barrier. In addition, owing to conceptual difficulties raised by Martin and Willis (2007), we avoided to calculate the relative contribution of individual barriers to total isolation. Overall, we found that total RI between the two species is strong (0.93), although none of the investigated barriers alone is sufficient to completely impede gene flow. Our results are thus consistent with an emerging pattern of multiple reproductive barriers acting together between closely related species (Lowry et al. 2008).

Out of the seven investigated barriers, we found that five mechanisms of RI contributed positively to the reduction of gene

flow between the two species (see Fig. 1). The main contribution to the maintenance of species boundaries is attributable to mechanisms that limit the exchange of interspecific pollen, such as those mediated by floral morphology, floral phenology (largely overlapping in this case; G. Scopece, pers. obs.), differential abundance of parental species and pollinator behavior that we estimated collectively through a synthetic index ($RI_{PRE\text{-}POLL}$). Although its role in plant speciation is still debated (Kay and Sargent 2009), this mainly pollinator-mediated isolation stage is thought to make a great contribution to the maintenance of sympatric plant species boundaries (Lowry et al. 2008) and is particularly important for specialized pollination systems (Xu et al. 2011). The two investigated species are instead known to exploit a weakly specialized food-deceptive pollination system attracting a wide range of insects in an unspecific manner (Schiestl 2005). Within this group it has been shown that even sympatric populations frequently share pollinators (Cozzolino et al. 2005). Our finding of strong prepollination isolation between closely related food-deceptive species with a similar pollinator set suggests that insect behavior, and in particular flower constancy (rather than specialization toward different pollinator species) may be an important reproductive isolating factor and represents an unexpected outcome that calls for more studies to disentangle the real causes of the reduced pollen exchange between weakly specialized plant species.

Postpollination prezygotic isolating mechanisms have been found to be strong among food-deceptive orchids (Scopece et al. 2007). However, the two investigated isolation stages (pollen–stigma incompatibility and conspecific pollen precedence) appear to be weak between *O. mascula* and *O. pauciflora*.

Postzygotic barriers contribute greatly to total isolation even if their contribution is masked by the early acting mechanisms. Although embryo mortality only weakly reduces gene flow between the two species, hybrid sterility appears to be stronger, which is consistent with the observed pattern of an earlier insurgence of hybrid sterility between related species (Scopece et al. 2008). In contrast, extrinsic postzygotic mechanisms appear to be weak with the hybrids that always occur in populations where the two parentals are also present and show pollination success that are comparable with the parentals suggesting that the mixing of parental genotypes does not lead to a breakdown of the phenotypic traits involved in insect attraction. Although the estimation of extrinsic postzygotic barriers would ideally be achieved with the help of experimental hybrids and the status of the hybrids (F1, backcross, F2, advanced generations) should be taken into account, our study was performed in natural populations. We used this approach because the study was conducted in an area (included in the Cilento e Vallo di Diano National Park) that has been deeply investigated and for which the dynamics of the hybrid zone are known in detail. For instance, the hybrid status of individuals included in this study was previously assessed by Cozzolino et al. (2008), who found, by molecular analyses, that approximately 70% of hybrids were F1s.

Only conspecific pollen precedence showed an asymmetric pattern between *O. mascula* and *O. pauciflora*. This pattern can potentially be caused by differences in style length (i.e., in gynostemium length in orchids; Kiang and Hamrick 1978; Sorensson and Brewbaker 1994). Molecular and morphological investigations of a hybrid zone between *O. mascula* and *O. pauciflora* showed an asymmetry in introgression patterns with a prominence of *O. pauciflora* genomes contributing to the hybrid genomes (Cozzolino et al. 2006). Our estimation of multiple components of RI shows a different pattern, that is similar levels of total isolation in the two species (0.94 in *O. mascula*, 0.95 in *O. pauciflora*; Fig. 1). This apparent incongruence is, however, not surprising because introgression patterns may be shaped by other factors such as the local density of species. Indeed, in similar cases (as is in the investigated sympatric zone where there is a higher density of *O. pauciflora*: G. Scopece, pers. obs.) the relative abundance of parental genomes is unbalanced, because it is more likely that first-generation hybrids will mate with the more abundant parent (Lepais et al. 2009).

Research carried out applying a case study approach cannot realistically describe all the potential mechanisms acting in limiting gene flow between closely related species because there are many mechanisms that potentially concur in this process and some of them are inevitably overlooked. Within these limitations, our survey of isolating mechanisms, which includes the most important stages of RI, suggests that in sympatry neither prepollination nor postpollination barriers may completely impede gene flow between the two species and that both make a comparable contribution (total strength of prepollination isolation = 0.78; total strength of postpollination barriers = 0.69; see Fig. 2). Similarly, integrity of species boundaries requires the joint action of both pre- and postzygotic mechanisms with the former making a slightly greater contribution to total isolation (total strength of prezygotic barriers = 0.80; total strength of postzygotic barriers = 0.66; see Fig. 2).

What does a similar architecture of RI tell us about the biogeographic condition during the early phases of species divergence? We refrain from speculating about the temporal order of the evolution of different mechanisms, because our quantification of isolating barriers represents a snapshot in evolutionary time and, as such, the current strength of barriers cannot necessarily be interpreted in terms of the timing of barrier evolution (Nosil et al. 2005). Nevertheless, the absence of a prominent type of isolation, which is in contrast with the generally emerging pattern of stronger pre- than postzygotic barriers (Lowry et al. 2008), strongly suggests that the speciation event required an allopatric condition.

Indeed, without a main (and quickly evolved) barrier, the homogenizing effect of gene flow in sympatry would overcome incipient species boundaries. Distribution data demonstrate that the two species have a clear eco-geographic separation (Delforge 2005). This different distribution pattern between the investigated species suggests that *O. pauciflora*, which has a restricted distribution (Delforge 2005), likely originated in more strictly Mediterranean ecological conditions during a period of geographic isolation likely due to paleoclimatic or geological events leading to habitat fragmentation or to insularity, which is a frequent condition in the Mediterranean basin (Thompson 2005). Nonetheless, even in sympatry, we showed that hybridization is strongly limited by pre- and postpollination isolating barriers and, when it occurs, postzygotic barriers impede the mixing of parental genomes. A similar picture suggests that speciation can start as a by-product of geographic isolation and that the causes of species formation can be an indirect consequence of population divergence in allopatry. Indeed, the inclusion of our results in the wider context of the orchid subtribe Orchidinae suggests that speciation in food-deceptive species may start as a consequence of the fixation of karyological differences (Cozzolino et al. 2004) such as those observed between *O. mascula* and *O. pauciflora* (D'Emerico et al. 2002). In contrast to our case study, in another species pair showing a comparable level of divergence at neutral traits (internal transcribed spacers, ITS: Aceto et al. 1999), it has been shown that in the absence of karyological differences, species boundaries remain permeable (Zitari et al. 2012). Karyological differences may be an important source of hybrid sterility (e.g., Lai et al. 2005) and the observation that chromosomal arms ratio and heterochromatin content were different between the two investigated species (D'Emerico 2001) suggests that the first event causing a reduction in the potential gene flow may be the fixation of chromosomal differences in allopatric populations (Cozzolino et al. 2004). In this context, the remaining barriers that have been found to contribute to the maintenance of species boundaries may have arisen secondarily as a by-product of genetic or ecological divergence or may have been actively selected in sympatry to avoid / limit the waste of gametes in the formation of hybrids with reduced fertility.

A similar scenario was also suggested by studies in the same orchid group carried out using the comparative method (Scopece et al. 2007, 2008; Cozzolino and Scopece 2008). Through the correlation of RI indices with genetic distances, the comparative approach contributed to the knowledge that, contrarily to prezygotic barriers that evolves erratically, postzygotic mechanisms evolves following a clock-like fashion and that hybrid sterility evolves faster than early acting postzygotic barriers (as hybrid or embryo inviability). Due to the limited number of barriers typically included in comparative studies, however, the contribution of individual components as well as the total strength of RI remained unclear. Using a case study approach allowed the identification of five mechanisms that positively impede gene flow between the investigated species and showed that the joint action of individual barriers is needed to gain the observed high value of total isolation. Furthermore, in the studies carried out using a comparative approach, the relative strength of prepollination versus postpollination and of prezygotic versus postzygotic barriers was investigated, but crucial aspects remained open (Scopece et al. 2007, 2009). This study, through a distinction of a larger number of isolating mechanisms, allowed a more precise understanding of the strength of prepollination versus postpollination and of prezygotic versus postzygotic barriers. The fine-scale identification of multiple barriers also allowed the identification of patterns that may be common in the orchid family such as the weakness of conspecific pollen precedence or the near absence of extrinsic postzygotic mechanisms. Thus, a combination of comparative and case studies emerges as an efficient way of addressing the evolution of RI during plant speciation.

Conclusion

Despite the absence of complete barriers, *O. mascula* and *O. pauciflora* are strongly isolated. We have shown that hybridization is strongly limited by pre- and postpollination isolating barriers and, when genetic contact occurs, late postzygotic barriers impede the mixing of parental genomes. Contrary to most surveys of isolating mechanisms in plants, our results speak against a clear predominance of prepollination or of prezygotic barriers. Rather, our results are consistent with the notion of multiple barriers contributing to RI among closely related species. More generally, they highlight the importance of similar studies to increase our knowledge of the evolution of RI and thus of the origin and maintenance of biological diversity in plants.

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