Evolution of Postzygotic Reproductive Isolation in a Guild of Deceptive Orchids

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ABSTRACT: The evolution of reproductive barriers is of central importance for speciation. Here, we investigated three components of postzygotic isolation—embryo mortality, hybrid inviability, and hybrid sterility—in a group of food-deceptive Mediterranean orchids from the genera *Anacamptis*, *Neotinea*, and *Orchis*. In these orchids, pollinator-mediated isolation is weak, which suggests that postpollination barriers exist. Based on crossing experiments and a literature survey, we found that embryo mortality caused complete reproductive isolation among 36.3% of the species pairs, and hybrid inviability affected 55.6% of the potentially hybridizing species pairs. Hybrid sterility was assessed experimentally for seven species pairs. A strong reduction of fertility in all investigated hybrids was found, together with clear differences between male and female components of hybrid sterility. Postzygotic isolation was found to evolve gradually with genetic divergence, and late postzygotic isolation (i.e., hybrid inviability and sterility) evolved faster than embryo mortality, which is an earlier postzygotic isolation stage. These results reveal that intrinsic postzygotic isolation strongly contributes to maintaining species boundaries among Mediterranean food-deceptive orchids while establishing a prominent role for these reproductive barriers in the early stage of species isolation.

Keywords: embryo mortality, food deception, hybrid inviability, hybrid sterility, *Orchis*, speciation.

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Speciation can start when barriers to gene flow evolve between formerly interbreeding demes and sufficiently restrict gene flow to overcome its homogenizing effect. Investigations of how, when, and why these gene flow barriers arise are important for our understanding of the speciation process.

Once species divergence has begun, the formation of a hybridogenic zygote represents a milestone in the series of events leading to genetic admixture of demes. Factors that prevent such zygote formation are often categorized according to whether they reduce the likelihood that heterospecific gametes will combine to form a viable zygote (prezygotic barriers) or reduce the viability or reproductive potential of interspecies hybrids (postzygotic barriers; Dobzhansky 1937; Tiffin et al. 2001). Whereas the former category is more strongly influenced by ecological and reproductive factors, the latter is highly dependent on the genetic constitution of species and is more influenced by intrinsic compatibility factors between parental species (Coyne and Orr 1989; Moyle et al. 2004).

In animals, hybrid sterility and inviability appear to evolve as described by the Dobzhansky-Muller model of incompatible alleles, and patterns characterizing the evolution of intrinsic postzygotic isolation (hybrid sterility and hybrid inviability) have been described in several animal systems, including flies (Coyne and Orr 1989, 1997), frogs (Sasa et al. 1998), birds (Price and Bouvier 2002), and butterflies (Presgraves 2002). In all these taxa, intrinsic postzygotic barriers, such as hybrid sterility and inviability, have been found to evolve gradually, giving rise to a "rough" speciation clock (Coyne and Orr 1989, 1997; Sasa et al. 1998; Orr and Turelli 2001; Presgraves 2002; Price and Bouvier 2002). Furthermore, hybrid sterility has been found to evolve more quickly than hybrid inviability in anurans (Sasa et al. 1998) and *Drosophila* (Coyne and Orr 1989).

In plants, these trends have not been firmly established. The association between postzygotic isolation and genetic distance in three angiosperm genera was found to range from largely positive to weak or even absent (Moyle et al. 2004). These associations were based on the results of

experimental crosses reported in the literature and consequently could not take into account the contributions and the strengths of the late postzygotic barriers such as hybrid viability and hybrid sterility. For instance, the finding in animals that hybrid male sterility evolves faster than female sterility or hybrid lethality has been attributed to the accelerated evolution of male traits via sexual selection/conflict (Wu et al. 1996); whether the same pattern also occurs in other groups, such as plants, that do not usually have separate sexes has not been tested.

Here, we investigate the evolution of early and late postzygotic reproductive barriers in a guild of nonrewarding, food-deceptive Mediterranean orchids. These orchids typically offer no reward to pollinators, have conspicuous flowers, and are thought to attract and deceive mostly naive pollinators by generally mimicking nectariferous plants (Cozzolino and Widmer 2005*b*). Mediterranean food-deceptive orchids have been found to display low pollinator specificity (Neiland and Wilcock 1999; Cozzolino et al. 2005), and they hybridize at low to moderate frequencies (Cozzolino and Widmer 2005*a*).

A recent experimental study quantified the strength of pre- and postpollination reproductive barriers among food- and sexually deceptive Mediterranean orchids but included only an early stage of postzygotic isolation (referred to as "postmating postzygotic isolation" in Scopece et al. 2007). However, little is known about the evolution in orchids of late postzygotic stages of reproductive isolation, such as hybrid sterility and hybrid inviability. Genetic analyses found that hybrid zones between food-deceptive orchids, in spite of their relative abundance, consisted mainly of first- or early-generation hybrids (reviewed in Cozzolino and Widmer 2005*a*). Factors shaping the genetic structure of these hybrid zones can be ascribed to a lower attractiveness of hybrid plants for pollinators of parental species (as described in *Ipomopsis*; Campbell et al. 1997) or to intrinsic genetic factors limiting hybrid reproductive success (as described in *Helianthus*; Quillet et al. 1995; Lai et al. 2005). Definition of the strength and the time course of the evolution of all the different kinds of reproductive barriers involved in species isolation is essential for understanding the speciation process (Coyne and Orr 2004).

We addressed the following questions in our study: (1) How much do the different stages of postzygotic isolation contribute to maintaining species integrity in a group of food-deceptive Mediterranean orchids? (2) Are the different stages of postzygotic isolation correlated with genetic distance? (3) What is the chronological order in which different components of postzygotic reproductive isolation evolve? (4) Do male and female sterility evolve at equal rates in these hermaphroditic orchids? To address these questions, we used a combination of literature surveys and experimental crosses involving natural hybrid individuals and parental species.

Methods

Postzygotic Isolation: Embryo Mortality

Data on embryo mortality among most species pairs examined in this study are taken from a study by Scopece et al. (2007), who compared fruit formation (prezygotic isolation) and embryo mortality (early postzygotic isolation) in experimental crosses in food- and sexually deceptive orchids. In this study, only data from species pairs among the food-deceptive genera *Anacamptis*, *Neotinea*, and *Orchis* (formerly all grouped into *Orchis* s.l.) are included from the data reported in Scopece et al. (2007). Additionally, new results that include crosses with *Anacamptis coriophora* (L.) R.M. Bateman, A.M. Pridgeon et M.W. Chase are included here. This species, together with its East Mediterranean vicariant *Anacamptis sancta* (L.) R.M. Bateman, A.M. Pridgeon et M.W. Chase, is the only rewarding species in the genus *Anacamptis* and is nested within a food-deceptive clade (Aceto et al. 1999; Bateman et al. 2003). The rewarding pollination thus appears to have evolved secondarily in this species (Cozzolino et al. 2001), which is known to be able to hybridize with related food-deceptive species of its clade.

Embryo mortality was defined as 1 − the proportion of viable embryos (i.e., viable seeds) produced in interspecific experimental pollinations, relative to the proportion of viable embryos produced in intraspecific crosses. That is, embryo mortality = 1 − (% viable seeds produced in interspecific crosses/% viable seeds produced in intraspecific crosses).

Postzygotic Isolation: Hybrid Inviability

The literature on the occurrence of orchids and their hybrids is vast, thanks to the great interest that this group has attracted from both scientists and laymen since Darwin's (1862) observations on the pollination of these plants. Using data from the literature allowed us to assess hybrid viability, an important component of postzygotic isolation that is difficult to estimate in an experimental approach because of the long generation time of orchids and the difficulty in growing large numbers of orchids from seeds.

All orchid species used for interspecific experimental crosses may easily occur in sympatry with overlapping flowering periods, and to assess hybrid inviability, we surveyed published reports on natural interspecific hybridization across all their distribution ranges (a complete list of references is available from G. Scopece on request).

Since orchid hybrids can be identified as hybrids only on flowering, we took reports of a particular hybrid combination as evidence of its viability and ability to survive at least until the initiation of flowering, which occurs normally after a period of two or more years under natural conditions (Arditti 1992). Because most reports on hybrids did not provide the necessary information, we refrained from estimating the frequency of hybrid formation or the number of discovered hybrid plants.

A potential source of error in literature data is the possibility that hybrid identifications, which typically are based exclusively on morphology, are erroneous and that putative hybrids may simply represent aberrant parental forms. This would lead to an overestimation of the number of species pairs that form viable hybrids in nature. At the same time, however, other factors may lead to an underestimation of the number of species pairs that form viable hybrids in nature. For instance, true hybrids can go undetected between species that have received little attention, particularly those from remote locations, or between species pairs with few or minor morphological differences, which obviously makes identification of hybrids based on morphology difficult (as in butterflies; see Presgraves 2002).

To reduce the number of false positives in our literature data set, we limited the records of hybrid reports to species pairs that effectively produced viable embryos in our experimental pollinations and whose gametes have some likelihood of coming in contact with each other under natural conditions (i.e., overlapping groups of pollinators; table A1 in the online edition of the *American Naturalist*). Within this subset of species, we tested for the presence of the hybrids in the literature, assuming that the absence of any hybrid report indicates hybrid inviability. Because of the lack of quantitative estimates of hybrid inviability, this trait was recorded as a binary trait, with 0 indicating hybrid viability and 1 indicating hybrid inviability.

Postzygotic Isolation: Hybrid Sterility

The study of F_1 hybrid fertility is also complicated by the long generation time of orchids and the difficulty in growing large numbers of orchids from seeds. However, because F_1 hybrids can be found in the field and their hybrid status can be tested with molecular markers (Pellegrino et al. 2001), these plants can be used to assess fertility.

Hybrid sterility was estimated experimentally by performing manual crossing experiments between hybrids and their parental species. Briefly, hand-pollination experiments were performed on plants that had been placed in cages covered with thin nets before flowering in order to prevent uncontrolled pollination. Pollination experiments were performed by touching the sticky viscidium with a plastic toothpick and removing pollinaria and placing them on the stigmas of other plants. Crosses between hybrids and backcrosses with parental species were performed bidirectionally, with each plant providing and receiving pollen.

Over a period of 6 years, the following hybrids were identified in the field and transferred to the Botanical Garden of Naples: *Anacamptis coriophora* × *Anacamptis laxiflora*, *Anacamptis morio* × *A. laxiflora*, *A. morio* × *Anacamptis papilionacea*, *Neotinea tridentata* × *Neotinea ustulata*, *Orchis anthropophora* × *Orchis simia*, *Orchis mascula* × *Orchis pauciflora*, and *O. pauciflora* × *Orchis quadripunctata* (see table 1 for taxon names and authors). To avoid errors in hybrid identification, we included only hybrid individuals for which the identities of the parental species and their F_1 status had been verified through molecular analyses (listed in Cozzolino and Widmer 2005*a*; S. Cozzolino, unpublished results). In detail, we performed 386 backcrosses between seven hybrid combinations (40 specimens) and 11 parental species (some parental species were involved in several hybrid combinations). Backcrosses with parental species were performed on a mean of 27.6 flowers (summing backcrosses performed in both possible directions for each of the parental species). On each hybrid combination, we performed bidirectional backcrosses; that is, individuals of each hybrid combination received pollen from both parents and also acted as pollen donors to each parent independently. This allowed us to estimate two components of hybrid sterility separately—male and female sterility. We defined the male component as having the capacity of hybrid pollen to induce fruit production and to successfully fertilize ovules that develop into viable seeds in the pollen-receiving parental plants. The female component was defined as having hybrid ovaries with the capacity to develop fruits and the ability to produce viable seeds when receiving pollen from parental species. The male and female components of hybrid sterility therefore consisted of two stages, fruit production (F) and viable seed production (S). Hybrid sterility indices were calculated separately for each stage and sex. The male fruit production isolation index was defined as the ratio of fruits produced in pollination attempts performed using hybrid pollen on both parental plants: $F_m = 1 -$ (number of fruits produced/number of pollinated flowers). Then the male seed production isolation index was estimated from the same crosses using the formula $S_m = 1 -$ (viable seeds/total number of counted seeds). The corresponding female isolation indices, F_f and S_f, were calculated using results from crosses in which the parental species were the pollen donors and the hybrids were the receiving (i.e., female) plants.

We then further calculated the linear combinations of the two stages in order to obtain a single value of hybrid

Table 1: Estimates of the strength of different stages of postzygotic isolation between hybridizing orchid species pairs

	Backcrosses							Crosses between hybrids		
	F_m	S_m	FS_m	F_f	S_f	FS_f	Hybrid sterility index	$H_{F1} \times H_{F1}$ *F*	$H_{F1} \times H_{F1}$ *S*	$H_{F1} \times H_{F1}$ *FS*
Anacamptis xparvifolia (Chaub.) R.M. Bateman, A.M. Pridgeon et M.W. Chase [2] (*Anacamptis laxiflora* × *Anacamptis coriophora*)	1 (20)	-	1	.08 (14)	1 (13)	1	1	1 (20)	-	1
Anacamptis xalata (Fleury) R.M. Bateman, A.M. Pridgeon et M.W. Chase [3] (*Anacamptis morio* × *A. laxiflora*)	1 (18)	-	1	.54 (18)	1 (8)	1	1	1 (8)	-	1
Anacamptis xgennari (Reichenbach. fil.) R.M. Bateman, A.M. Pridgeon et M.W. Chase [5] (*A. morio* × *Anacamptis papilionacea*)	1 (28)	-	1	.14 (30)	.99 (26)	.99	1	.91 (11)	1 (1)	1
Neotinea xdietrichiana (Bogenh.) R.M. Bateman, A.M. Pridgeon et M.W. Chase [2] (*Neotinea ustulata* × *Neotinea tridentata*)	.28 (12)	.54 (9)	.67	0 (9)	.20 (9)	.20	.43	0 (5)	.68 (5)	.68
Orchis xcolemanii Cortesi [24] (*Orchis mascula* × *Orchis pauciflora*)	.10 (67)	.53 (60)	.58	.10 (55)	.41 (49)	.47	.52	.10 (22)	.82 (20)	.84
Orchis xpseudoanatolica Fleishm. [2] (*O. pauciflora* × *Orchis quadripunctata*)	1 (7)	-	1	.29 (7)	.99 (5)	.99	1	1 (6)	-	1
Orchis xbergonii Nanteuil [2] (*Orchis simia* × *Orchis anthropophora*)	1 (48)	-	1	.06 (53)	1 (50)	1	1	1 (7)	-	1
Isolation index mean value	.77	.54	.89	.17	.80	.81	.85	.72	.83	.93

Note: Male (subscript m), and female (subscript f) indices for hybrid sterility at fruit (*F*) and seed (*S*) stages were estimated from experimental crosses with natural hybrids. Brackets enclose the numbers of hybrid specimens; parentheses enclose the numbers of pollination attempts. Hyphen = not estimable.

sterility for each sexual component: $FS_m = F_m + (1 - F_m) \times S_m$ and $FS_f = F_f + (1 - F_f) \times S_f$, as done by Moyle et al. (2004). Accordingly, the mean of the two combined values represents our hybrid sterility index, mean $FS = (FS_m + FS_f)/2$.

The capacity of hybrid individuals to produce viable F_2 seeds was also tested by crossing different F_1 hybrid individuals (H_{F1}) of the same parental combination as described above. The results were used to estimate two isolation indices for the different stages: $H_{F1} \times H_{F1}$ $F =$ 1 − (number of fruit produced/number of flowers pollinated) and $H_{F1} \times H_{F1}$ $S = 1 -$ (% viable seeds). Then the linear combination of these two indices was $H_{F1} \times H_{F1}$ $FS = H_{F1} \times H_{F1}$ $F + (1 - H_{F1} \times H_{F1}$ $F) \times H_{F1} \times H_{F1}$ S.

Correlation Between Reproductive Isolation and Genetic Distance

An estimate of genetic distances between species pairs was calculated by using nuclear internal transcribed spacer sequences. Briefly, the sequences, which were all produced in our previous studies (Aceto et al. 1999; Cozzolino et al. 2001) and are available in GenBank, were hand-aligned in Bioedit (Hall 1997), and the best-fit model of molecular evolution (TrN + G) was estimated with ModelTest 3.7 (Posada and Crandall 1998). Genetic distances were then calculated in PAUP* 4.0 (Swofford 1999).

Due to the qualitative (presence/absence) nature of data of hybrid inviability, to test for associations between this isolation stage and genetic distances, we performed a binary logistic regression. For the other two stages of postzygotic isolation, embryo mortality and hybrid sterility, we performed standard correlation analyses with genetic distances between parental species, as done by Moyle et al. (2004). Because some of the parental species were involved in more than one hybrid combination and some species were involved in multiple crosses, a number of data points within our data sets were not statistically independent. To make sure that the observations were phylogenetically and statistically independent, we selected strictly independent species pairs in order to maximize the number of pairs that could be obtained from the available nonindependent data sets (Felsenstein 1985). Because the genetic distance and isolation data were not normally distributed, we employed the nonparametric Kendall's τ rank correlation for full and strictly independent data sets, using SPSS 13.0 (SPSS, Chicago) to test for associations.

Relative Evolutionary Rates of Postzygotic Isolation

Although we have no direct calibration of evolutionary rates in our group of orchids, we used genetic distances as a proxy for time since divergence to examine relative rates of evolution of each stage of reproductive isolation (Coyne and Orr 1989, 1997; Sasa et al. 1998; Presgraves 2002). To compare relative rates of evolution of the three stages of postzygotic isolation, we compared the average genetic distances of the species pairs displaying total isolation for a given postzygotic isolation index (Coyne and Orr 1989, 1997; Sasa et al. 1998; Presgraves 2002). To do so, therefore, we simply evaluated the presence/absence of each stage of postzygotic isolation (embryo mortality, hybrid sterility, hybrid inviability). Since our data were not normally distributed, to compare mean genetic distances among the three stages, we used the nonparametric Kruskal-Wallis test with a Mann-Whitney *U*-test for a posteriori multiple comparison, with the significance level set to .01 (Bonferroni correction).

Instead of noting only the presence/absence of hybrid sterility and embryo mortality for these two stages, we gathered quantitative data (i.e., our isolation indices). Because for these two stages we were more interested in the beginning of speciation and because reproductive isolation must reach an asymptote at higher genetic distances, we followed Coyne and Orr (1989) and limited our comparison to those species pairs separated by low genetic distances ($\geq$0.25; this value was chosen arbitrarily, but lower thresholds produce qualitatively similar results). Within this interval, we performed a Mann-Whitney *U*-test to assess significant differences in the strength of the two stages of isolation.

For seven species pairs, we gathered data for embryo mortality and hybrid sterility. For these species pairs, the mean rate at which isolation evolved was estimated as the linear regression coefficient of the reproductive isolation on the genetic distance. The regression was constrained through the origin because we assumed that species start off as populations that are genetically identical and reproductively compatible (Hillis et al. 1996).

Results

Postzygotic Isolation: Embryo Mortality

From the 136 interspecific crosses performed among the 17 orchid species, 23 crosses produced no fruits, due to complete isolation from postpollination prezygotic barriers (Scopece et al. 2007). These species pairs were thus excluded by the evaluation of embryo mortality that was estimated on the remaining 113 interspecific crosses that produced fruits (containing viable or unviable seeds). Complete embryo mortality was found in 41 combinations (36.3%) of these 113 interspecific crosses (table A1).

Postzygotic Isolation: Hybrid Inviability

For the estimation of hybrid inviability by using literature data, several species combinations were removed from the original data set of 136 interspecific crosses. Our crossing experiments indicated no hybrid formation (because no fruit and no viable seeds were produced) and, thus, no further estimation of hybrid inviability for 64 species pairs was possible. In detail, we excluded the 23 interspecific crosses that produced no fruits and the 41 combinations that, due to complete embryo mortality, produced no viable seeds. Then, we also excluded 14 species pairs that are known to be pollinated by different insect groups (Van der Cingel 1995; Scopece et al. 2007) and for which the absence in the literature of reported hybrids is probably a consequence of strong prepollination isolation between species rather than an indication of embryo mortality. Finally, due to the complete lack of pollinator information for *Neotinea lactea* (Poir.) R.M. Bateman, A.M. Pridgeon et M.W. Chase, we cannot establish whether any pollinator barriers may prevent hybrid formation with this species, so we also excluded the 13 species pair combinations in which *N. lactea* was involved (table A1). As a consequence of all these exclusions, hybrid inviability was assessed for 45 species pairs only.

Hybrid occurrence has been reported in the literature for 20 out of the 45 species pairs. For 25 species pairs, no hybrids have been reported from natural populations, indicating that 55.6% of the 45 species pairs are affected by some sort of hybrid inviability.

Postzygotic Isolation: Hybrid Sterility

For estimation of hybrid sterility by using experimental crosses, we used seven hybrid combinations. Hybrid sterility was assessed by experimental crosses between hybrid individuals and their parental species. In these backcrosses, we found a strong reduction of fertility in all the investigated hybrids. Only two (*Orchis mascula* × *Orchis pauciflora* and *Neotinea tridentata* × *Neotinea ustulata*) of the seven experimentally examined hybrids, when backcrossed with parental species, produced some fruits and viable seeds (more than 1%), whereas all other hybrids were entirely (*Orchis anthropophora* × *Orchis simia*, *Anacamptis morio* × *Anacamptis laxiflora*, *A. morio* × *Anacamptis papilionacea*, *Anacamptis coriophora* × *A. laxiflora*) or almost entirely (*O. pauciflora* × *Orchis quadripunctata*) sterile. The mean F_m was 0.77, the mean S_m was 0.54, and the hybrid sterility index was 0.85 (table 1).

These experiments further revealed a significant difference between male and female components of hybrid sterility. Hybrid pollen were able to trigger the development of fruits in parental species in only two cases (*O. mascula* × *O. pauciflora* and *N. tridentata* × *N. ustulata*). However, in these two cases, seed viability was strongly reduced when compared with values of the intraspecific crosses of the parental species. Seed production in parental species with *O. mascula* × *O. pauciflora* hybrid pollen was 47.2% (92.8% in intraspecific *O. mascula* crosses; 85.5% in *O. pauciflora*). Similarly, seed production in parental species with *N. tridentata* × *N. ustulata* hybrid pollen was 46.5% (56.5% in intraspecific *N. tridentata* crosses; 62.9% in *N. ustulata*).

The situation was different when the hybrids received pollen from the parental species. In this case, fruit production was high (82.7%; mean $F_f = 0.17$), but again, seed viability was strongly reduced (20.0%; mean $S_f = 0.80$) compared with intraspecific crosses between the parental species.

Therefore, both the male and female components of hybrid sterility strongly contributed to reproductive isolation at this stage (mean value of FS_m index $= 0.89$; mean value of FS_f index $= 0.81$) but in different ways. The male component led to low fruit production, whereas the female component led to low seed viability (see fig. 1).

Crosses between F_1 hybrid individuals to produce F_2 embryos also resulted in a lower fruit production (28.6%; mean $H_{F1} \times H_{F1}$ *F* index $= 0.72$) and lower seed viability (16.6%; mean $H_{F1} \times H_{F1}$ *S* index $= 0.83$) than corresponding backcrosses. Only the hybrids *O. mascula* × *O. pauciflora* and *N. tridentata* × *N. ustulata* were able to produce F_2 seeds in experimental crosses. For each hybrid, the combined value of the isolation indices for the two stages was $H_{F1} \times H_{F1}$ *FS*. The average of all $H_{F1} \times H_{F1}$ *FS* indices was 0.93 (see table 1).

Correlation Between Reproductive Isolation and Genetic Distance

In the 45 species pairs used to estimate hybrid inviability, we found a significant correlation by logistic regression ($r^2 = 0.557$, $F = 55.39$, $P < .001$, regression coefficient $= 0.17$) between this stage of isolation and genetic distance, which we used as a proxy for the time since species diverged. However, our qualitative estimates of hybrid inviability, based on hybrid presence/absence, did not allow us to evaluate the strength of this correlation.

From inspection of the embryo mortality data set (including a total of 113 species pairs), we found a positive and strongly significant association between this stage of postzygotic isolation and genetic distance (Kendall's $\tau = 0.623$, $P < .001$). This correlation was also significant in the strictly independent species pairs (Kendall's $\tau = 0.571$, $P = .048$; see table 2).

Hybrid sterility was also found to be positively correlated with genetic distance in both the full data set (seven

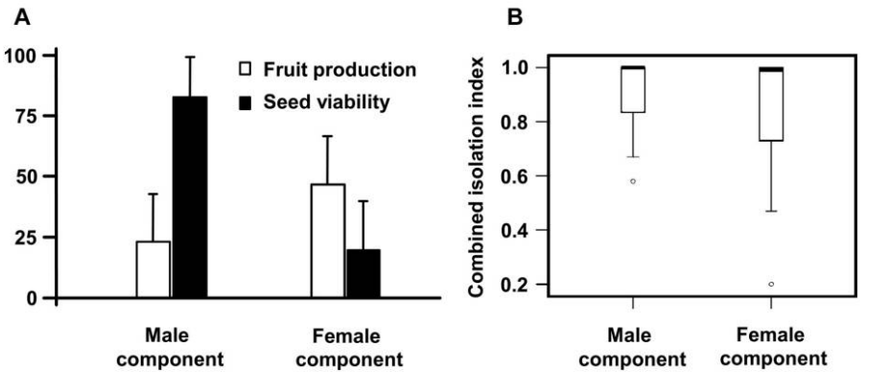

Figure 1: Differences between male and female components of hybrid sterility. *A*, Fruit production and seed viability in crosses in which hybrid individuals were the pollen donor (male component) or the ovule donor (female component); *B*, combined male and combined female indices. Circles represent outlying crosses.

hybrid combinations, Kendall's $\tau = 0.724$, $P = .037$) and the strictly independent data set (five hybrid combinations, Kendall's $\tau = 0.837$, $P = .052$; see table 2). Although the hybrid sterility data set was only a subsample of the literature-based data set on reported hybrids, it nevertheless represents 35% of the known cases (i.e., seven out of 20 hybrid combinations reported in literature).

Relative Evolutionary Rates of Postzygotic Isolation

We found that the strength of all three estimated stages of postzygotic isolation increased with genetic distance and, thus, with time since divergence between species (fig. 2). We found significant differences ($\chi^2 = 24.121$; $P < .001$; see fig. 2) among average genetic distances at which the different stages of postzygotic isolation completely evolved. Specifically, species pairs showing complete hybrid sterility were significantly less genetically divergent than those showing complete embryo mortality (Mann-Whitney U-test = 12.0; $P < .001$). Similarly, species pairs showing complete hybrid inviability were significantly less genetically divergent than those showing complete embryo mortality (Mann-Whitney U-test = 187.5; $P < .001$). We did not find any significant difference in the average genetic distance between species pairs showing complete hybrid sterility and hybrid inviability (Mann-Whitney U-test = 48.0; $P = .419$).

When we compared embryo mortality and hybrid sterility among the less divergent species (those falling in the interval of interspecific genetic distance ≥ 0.25), our comparison showed significantly higher hybrid sterility than embryo mortality (Mann-Whitney U-test = 55.00; $P = .017$). This result was not an artifact of a different average genetic distance between the embryo mortality and the hybrid sterility data set. In fact, within the selected interval, the average genetic distance among the species pairs was nearly identical in both data sets (Mann-Whitney U-test = 112.50; $P = .465$). Similarly, when we compared the strength of embryo mortality and of hybrid sterility for each of the seven species pairs for which we gathered both isolation indices, all but one (*N. ustulata* × *N. tridentata*) displayed a hybrid sterility index higher than the embryo mortality index (see table A1).

By considering these seven species pairs, we also found a positive correlation between reproductive isolation indices and genetic distance (embryo mortality: $r^2 = 0.798$, $F = 23.6$, $P = .003$; hybrid sterility: $r^2 = 0.750$, $F = 17.99$, $P = .005$) and a higher regression coefficient for the hybrid sterility index (regression coefficient 4.480) than for the embryo mortality index (regression coefficient 2.779; see fig. 3).

Discussion

A prominent role for hybridization in creating new orchid species has repeatedly been emphasized in the past (e.g., Van der Pijl and Dodson 1966; Ehrendorfer 1980), and our literature survey at first glance supports this notion. All 17 species of Mediterranean food-deceptive orchids considered in this study hybridize to some degree in nature. However, all naturally hybridizing species pairs for which we could experimentally assess hybrid sterility (11 species, or 64.7% of investigated species) displayed moderate to strong intrinsic hybrid fitness problems. With the exception of the two least genetically divergent species pairs, all examined hybrids were practically sterile and had a hybrid sterility index >0.95. But hybrid sterility does not appear to be the only prerequisite for the coexistence of

species in sympatry. Embryo mortality, an earlier stage of isolation, prevents any production of viable hybrid seeds in 36.3% of species pairs included in our data set. This estimation, performed on 17 species belonging to the food-deceptive-related *Anacamptis*, *Orchis*, and *Neotinea* genera (formerly all included in *Orchis* s.l.) and also including the rewarding *Anacamptis coriophora*, closely matches the corresponding value of 36.6% reported by Scopece et al. (2007), who compared rates of pre- and postpollination isolation between sexually deceptive and food-deceptive orchids. Thus, this isolation mechanism plays an important role in maintaining isolation among the sympatric food-deceptive orchids examined in this study. Overall, our results reveal that natural hybridization is widespread among Mediterranean food-deceptive orchids but that strong intrinsic postzygotic isolation mechanisms secure species boundaries among sympatric species that have low pollinator isolation.

Lethality of F_1 hybrids after germination has been reported in many plant genera, with hybrid weakness and dwarfs as common features (Levin 1978; reviewed in Bomblies and Weigel 2007). We estimated that 55.6% of our 45 potentially hybridizing orchid species pairs are affected by some sort of hybrid lethality because they produced viable embryos when manually crossed, but viable hybrids in natural populations have never been recorded in the literature. Because hybrid reports in the literature are based on morphological identification of flowering hybrids, we cannot distinguish between hybrid lethality occurring at an early developmental stage, such as seed germination or seedling establishment, and that at a later stage (with hybrid plants unable to reach the reproductive phase). Also, we cannot distinguish between intrinsic and extrinsic causes of hybrid inviability. However, all hybrid plants reported in the literature or observed directly in the field in this study were as healthy and vigorous as the parental plants. Moreover, parental species often grow intermingled with each other at many sites and display no obvious habitat differences, which may indicate that the extrinsic causes of hybrid inviability are unlikely. More probably, the most critical stages in the life cycle of orchids are the seedling stages, because an intimate association with an appropriate mycorrhizal partner must be established for survival (Gardes 2002). The genetic basis of this association and the mechanisms of cellular signaling among orchid seedlings and fungal partners remain to be investigated (McCormick et al. 2004; Shefferson et al. 2005), and it is unknown whether the specificity of this interaction may prevent hybrid seeds from the successful establishment of a fungal association.

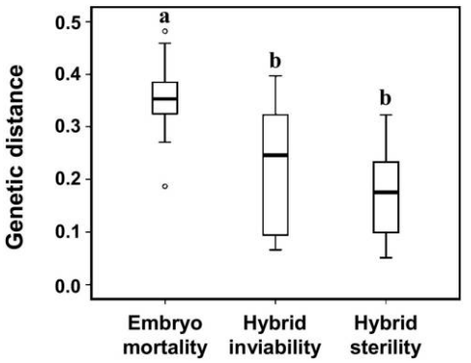

Figure 2: Relative strength of postzygotic isolation. Comparison of average genetic distance between species pairs displaying total isolation for a given postzygotic isolation index. (Different letters indicate significant differences; Mann-Whitney *U*-test, $P < .01$ after Bonferroni correction). The corresponding species pair data sets are reported in table A1 in the online edition of the *American Naturalist*.

When viable F_1 hybrids are formed, sterility or reduced reproductive output may result in a major barrier to gene flow between these food-deceptive orchids. This observation is consistent with those in other plants, in which hybrid sterility seems to be an important factor in reproductive isolation (Levin 1978). In our study, we found that only two hybrid combinations were able to partially produce viable seeds when backcrossed with parental plants, whereas all other hybrids were almost or completely sterile both when backcrossed with parental species and when crossed with other hybrids.

In contrast to prezygotic isolation, which seems to arise in a less predictable way (Gleason and Ritchie 1998; Panhuis et al. 2001), postzygotic isolation has been found to evolve gradually with genetic distance in all animal taxa

Table 2: Correlation between postzygotic isolation and genetic distance

	Embryo mortality			Hybrid sterility		
Data set/analysis	*N*	Correlation	*P*	*N*	Correlation	*P*
Full data set	113 (17)	.623	<.001	7 (11)	.724	.037
Strictly independent species pairs	8 (16)	.571	.048	5 (10)	.837	.052

Note: *N* = number of species pairs; parentheses enclose number of species included in the data set; Correlation = Kendall's test; *P* = significance value.

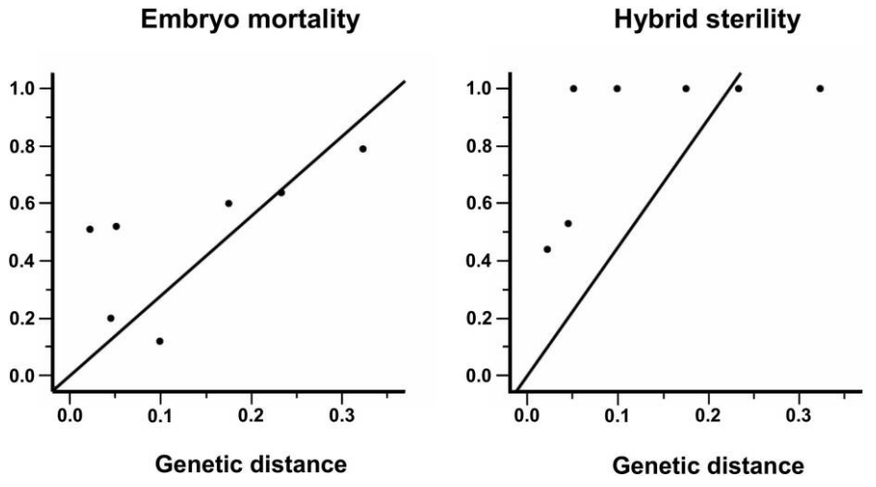

Figure 3: Hybrid sterility and embryo mortality versus genetic distance. The scatterplots represent the correlation of genetic distance with hybrid sterility and embryo mortality for the seven species pairs for which we gathered both isolation indices.

that have been investigated (Coyne and Orr 1989, 1997; Sasa et al. 1998; Presgraves 2002). In this study, we found that postzygotic isolation evolves gradually with genetic divergence at each of the three investigated stages and that late postzygotic isolation (i.e., hybrid inviability and sterility) evolves faster than early postzygotic isolation (i.e., embryo mortality). Similar patterns have been reported in many animal taxa (Wu 1992; Sasa et al. 1998; Presgraves 2002; Price and Bouvier 2002). Regarding late postzygotic isolation factors, due to the contrasting nature of our data (qualitative/quantitative), we could not estimate whether hybrid sterility evolved faster than hybrid inviability or vice versa.

Because most animals have separate sexes—in contrast to most flowering plants—male and female hybrid sterility are often considered separately. Typically, animal hybrids perform as predicted by Haldane's rule, which posits that hybrids of the heterogametic sex are more strongly affected by sterility than those of the homogametic sex (Coyne and Orr 2004). In animals, the typical chronology of the evolution of postzygotic isolation mechanisms therefore is male hybrid sterility, female hybrid sterility, and hybrid inviability (Christianson et al. 2005). We estimated the male and female components of reproductive isolation separately in this study. In the two hybrid combinations that were partially fertile, we did not find a significant difference in the strength of reproductive isolation between the male and female components (see fig. 1*b*), but we found a marked difference in their modality (see fig. 1*a*), as previously reported for Australian (Peakall et al. 1997) and tropical orchid hybrids (Stort 1984). This difference is most likely a consequence of the fact that in orchids, female gametophyte development is induced by biochemical signals that are elicited by the arrival of compatible pollen on the stigma (Zhang and O'Neill 1993). The low fruit production following backcrosses with F_1 hybrids serving as pollen donors indicates that F_1 hybrid pollen is of low quality and cannot induce the development of the female gametophyte in parental plants. On the contrary, when parental species are used as pollen donors on F_1 hybrids, their much more viable pollen successfully promote fruit formation in hybrid plants. In both crossing directions, however, we have observed reduced seed viability in backcrosses compared to intraspecific crosses. As expected, crosses between F_1 hybrid plants, where both male and female sterility components interact, produced even fewer fruits and seeds than backcrosses.

Sterility of F_1 hybrids can be produced by genetic incompatibilities and/or chromosomal mutations. Empirical work in *Drosophila* has revealed that hybrid fertility problems are often highly polygenic and complex (reviewed in Coyne and Orr 2004), that a large number of loci interact negatively in the hybrid genetic background, and that these interactions act cumulatively to cause inviability and sterility because of the fixation of incompatible alleles, as predicted by the Dobzhansky-Muller incompatibility model (Wu and Palopoli 1994; Rieseberg and Carney 1998). However, even just a few loci can cause hybrid sterility, as observed in the plant genus *Mimulus*, in which nearly complete hybrid male sterility may result from a simple genetic incompatibility between a single pair of heterospecific loci (Sweigart et al. 2006; Martin and Willis 2007). Similar examples exist in other plant species, in which a small number of loci have a major effect on hybrid

sterility and inviability (reviewed in Bomblies and Weigel 2007).

In addition to genic causes of incompatibility, chromosomal rearrangements also play a critical role in hybrid sterility, but the evolutionary dynamics associated with chromosomal changes are expected to be quite different from those associated with Dobzhansky-Muller incompatibilities (Archibald et al. 2005). In crosses between chromosomally divergent species, sterility can be attributed to the effects of chromosomal rearrangements on meiotic pairing. In genus *Helianthus* for example, several species appear to differ by one or more chromosomal translocations, and these chromosome rearrangements are largely responsible for reducing pollen viability of F_1 hybrids (Quillet et al. 1995; Rieseberg and Carney 1998; Rieseberg et al. 1999; Lai et al. 2005). Similarly, in our guild of food-deceptive orchids, most species have divergent karyotypes (D'Emerico et al. 2002). The strong hybrid sterility detected in our study could be a consequence of the karyological differences among parental taxa, because such differences can lead to problems during chromosome pairing in hybrids (Cozzolino et al. 2004).

Chromosomal rearrangements have strongest effects on late postzygotic isolation mechanisms such as F_1 seeds and pollen viabilities (Stebbins 1971; Rieseberg 2001) but do not affect earlier stages of postzygotic isolation, such as embryo mortality, that are more strongly influenced by the gradual accumulation of incompatibilities among parental taxa (Orr and Turelli 2001) and therefore (when multigenic) may need more time to arise.

For the species pairs for which we were able to estimate all stages of reproductive isolation, both embryo mortality and hybrid sterility were correlated with genetic distance (see fig. 2). At lower genetic distances, we found stronger isolation for hybrid sterility than for embryo mortality, suggesting that hybrid sterility may evolve faster than embryo mortality. This result is coherent with the proposed role of chromosome rearrangements as the primary cause of reproductive isolation among food-deceptive orchids and could explain the existence of partial hybrid fertility only in the two most closely related species pairs investigated (*Neotinea ustulata* × *Neotinea tridentata* and *Orchis mascula* × *Orchis pauciflora*). Once hybrid sterility has evolved between two species, natural selection may promote the evolution of prepollination reproductive barriers in secondary contact zones, such as floral isolation or heterospecific pollen/female gametophyte interaction, but not evolution of other postzygotic isolation barriers, because for the parental species, costs are incurred by the wasteful use of gametes.

A recent study by Moccia et al. (2007) assessed the genetic structure of independent hybrid zones between *Anacamptis morio* (L.) R.M. Bateman, A.M. Pridgeon et M.W. Chase and *Anacamptis papilionacea* (L.) R.M. Bateman, A.M. Pridgeon et M.W. Chase and found no evidence for introgression but strong hybrid sterility between these frequently hybridizing species. Their results are thus concordant with experimental results in our controlled crosses and suggest that intrinsic postzygotic isolation mechanisms effectively contribute to reproductive isolation between sympatric food-deceptive orchid species in nature and are implicated in the early stages of species isolation and speciation. These conclusions challenge the widely held view that postzygotic isolation is of little relevance to speciation in orchids (Gill 1989) and highlight the importance of detailed comparative analyses in understanding the evolution of reproductive isolation and speciation.

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