

Evidence for reproductive isolate selection in Mediterranean orchids: karyotype differences compensate for the lack of pollinator specificity

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Mediterranean orchids of the subtribe Orchidinae are highly diverse and display a range of fascinating pollination strategies. Based on observations that orchid–pollinator relationships are often highly specialized and species specific, Darwin and others have argued that selection for different pollinators has been the driving force behind the evolutionary diversification of orchids. This may be true for orchids that attract different, specialized pollinators that act as prezygotic reproductive barriers. It is, however, not clear how closely related co-flowering Mediterranean orchids that share pollinators survive the challenge of sympatry. We show that species pairs with a generalized pool of pollinators have significantly more divergent karyotypes compared with species pairs with different pollinators. These results show that karyotype differences that act as postzygotic reproductive barriers may have played an important role in the evolution of Mediterranean orchid diversity.

Keywords: karyotype evolution; orchids; pollination; reproductive isolation; speciation

1. INTRODUCTION

With 788 genera and *ca.* 18 500 species, orchids are among the most species-rich plant families and display an extraordinary floral diversity.

Orchid flowers are often highly specialized to attract and fit their pollinators, and many orchid–pollinator relationships are species specific (Van der Pijl & Dodson 1966). In this case, pollinator specificity may act as an ethological, prezygotic reproductive isolation mechanism among orchid species that grow sympatrically and have overlapping flowering phenologies (Van der Pijl & Dodson 1966; Dressler 1993). The observations that experimental crosses are possible among many orchid species and that hybrids are relatively rare between sympatric orchid species seem to support the role of pollinator specificity in

maintaining orchid species boundaries (Darwin 1862; Van der Pijl & Dodson 1966). A large role has thus been given to prezygotic reproductive isolation mechanisms in orchids, whereas postzygotic isolation mechanisms in orchids are typically ignored.

However, orchid pollination is not always species specific. A compilation of pollinator information for European orchids by Van der Cingel (1995) reveals that species-specific orchid–pollinator relationships have evolved in only a few groups, such as the sexually deceptive genus *Ophrys* (Schiestl *et al.* 1999), whereas many orchid species are generalists with respect to the choice of their pollinators. A high proportion of European species have large conspicuous flowers that offer no reward to their pollinators and are known as food-deceptive orchids (Dafni 1984). Some of these species mimic nectariferous flowers that grow in the same habitat, a phenomenon that is known as Batesian floral mimicry, whereas others attract and deceive naive pollinators that respond to the large showy flowers, nectar spurs and nectar guides. These orchid species often attract a diverse range of pollinators that may visit several different food-deceptive orchid species (Dafni 1984; Roy & Widmer 1999; Widmer *et al.* 2000).

To maintain species boundaries in orchid species that share pollinators, closely related species should either have allopatric distribution areas, have non-overlapping flowering periods or have reproductive isolation mechanisms other than pollinator specificity.

A well-supported phylogenetic framework for the European Orchidinae exists (Bateman *et al.* 1997, 2003; Aceto *et al.* 1999; Cozzolino *et al.* 2001). A comparison of the geographical distribution areas of species pairs (not necessarily sister species) identified in these phylogenies reveals that many species pairs have overlapping distribution areas. Moreover, many species pairs may grow in sympatry, i.e. within a few metres of each other in many localities, and have overlapping flowering phenologies. Contrary to expectations, such species pairs not only include taxa that attract different, specialized pollinators, but also include species pairs that share a common pool of unspecific pollinators (Van der Cingel 1995).

We ask the question how Mediterranean orchid species pairs that grow in sympatry, have overlapping flowering periods and share pollinators, survive the challenge of sympatry? We propose that pollinator specificity is not sufficient to explain species diversity in these European Orchidinae and that chromosomal differences aid in the maintenance of species boundaries in sympatric, co-flowering species with overlapping pollinator pools.

To address this problem, we estimated the level of karyotype divergence between members of species pairs to test the hypothesis that there is no difference in karyotype divergence between species pairs that either share a pool of pollinators or are pollinated by different, specific pollinators.

2. MATERIAL AND METHODS

Using previously published phylogenetic analyses of European Orchidinae, we selected a total of 13 species pairs that may grow in sympatry, have overlapping flowering periods and for which pollinator information was available. We identified six species pairs that share a pool of pollinators, and seven species pairs that attract different, specific pollinators (figure 1).

Karyotype information was either taken from the literature (D'Emérico 2001; D'Emérico *et al.* 2002a) or is reported here for the first time, to our knowledge (table 1). Mitotic and meiotic chromosomes were prepared from immature ovaries as described in

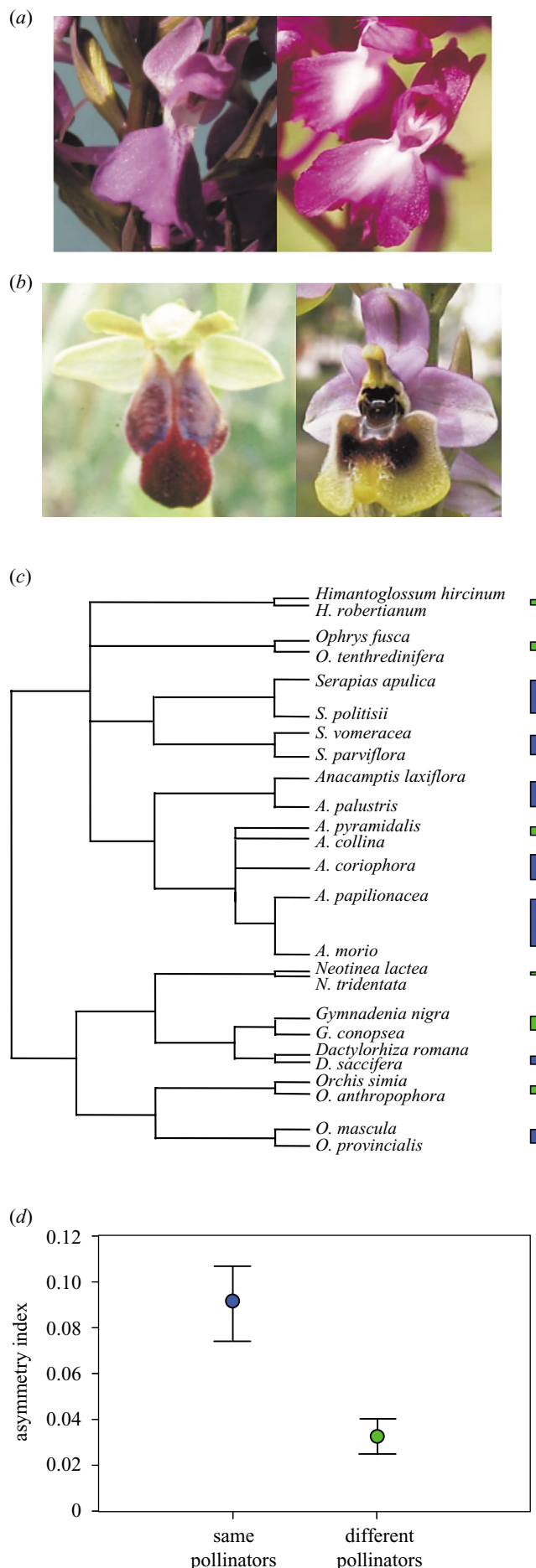


Figure 1. Chromosomal divergence and floral diversification in European Orchidinae. (a) *Anacamptis palustris* and *A. laxiflora* share a common pool of pollinators. (b) *Ophrys fusca* and *O. tenthredinifera* have different, species-specific pollinators. (c) One of the selected internal transcribed spacer's (ITS) trees showing the phylogenetic relationships among species pairs of Mediterranean Orchidinae used in this study. The length of the vertical bars is proportional to the intrachromosomal asymmetry index difference between species pairs (blue: species share a common pollinator pool; green: species have different pollinators). (d) Mean and standards errors of intrachromosomal asymmetry index differences (absolute values) between species pairs that share pollinators (blue) or have different pollinators (green).

D'Emérico *et al.* (2002a). For each sample, arm length was estimated for all chromosomes in each karyotype and for every pair of chromosomes the average arm length was calculated. For each species we calculated the intrachromosomal asymmetry index (AI) according to Zarco (1986). This index is a quantification of Stebbins's asymmetry categories. It ranges between 0 and 1 and is low when chromosomes tend to be metacentric. This index is independent of chromosome number and size and is used here to estimate the level of karyotype divergence between members of each species pair.

Because orchid species pairs are not independent as a consequence of their shared phylogenetic history, we took phylogenetic relationships among species pairs into consideration (Martins & Hansen 1997). A phylogenetic analysis of nuclear ribosomal ITS1 and 2 sequences of the selected taxa was carried out as described in Cozzolino *et al.* (2001). A phylogenetic regression of the intrachromosomal asymmetry index (y) on pollinator identity (x) was carried out using the phylogenetic generalized least-squares regression (PGLS) method as implemented in the software package COMPARE (available at <http://compare.bio.indiana.edu>). PGLS can be viewed as a method that allows for flexibility in the underlying evolutionary assumption through the use of a single parameter (α) that represents a measure of evolutionary constraints acting on a trait. When α is large, comparative data are independent of phylogeny. We coded 'different pollinators' as $x = 1$ and 'shared pollinators' as $x = 0$, and assumed that there was no within-taxon variation. To test whether data were normally distributed we used the Kolmogorov–Smirnov test implemented in SPSS (SPSS, Inc.).

To determine whether our estimates of karyotype divergence are affected by the genetic distance between members of species pairs, we estimated pairwise sequence comparison (in terms of percentage of bases differences) for members of each species pair using previously published nuclear ribosomal ITS1 and 2 sequences (Aceto *et al.* 1999; Cozzolino *et al.* 2001; Soliva *et al.* 2001) (see table 1 for GenBank accession numbers). We then tested for a correlation between genetic distance and karyotype divergence using SPSS (SPSS, Inc.).

3. RESULTS

We found a higher mean intrachromosomal asymmetry index difference between species pairs that share a pool of pollinators (mean = 0.089, s.e. = 0.017), compared with species pairs that have different pollinators (mean = 0.028, s.e. = 0.0054). Intrachromosomal asymmetry index differences were normally distributed (Kolmogorov–Smirnov test, $p = 0.200$). The PGLS analysis revealed that intrachromosomal asymmetry index differences were significantly different between the two groups (RE = -0.6 , s.e. = 0.02), and were independent of phylogeny, as indicated by a large value of α ($\alpha = 15.5$).

Similar results have been obtained by using the topologies reported in Cozzolino *et al.* (2001) and Bateman *et al.* (2003), which differ in the position of *Neotinea* relative to the other genera.

Karyotype divergence was not significantly correlated with genetic divergence between members of species pairs (Spearman's $r_s = -0.111$, $p = 0.72$).

Table 1. Chromosome numbers, intrachromosomal asymmetry indexes and GenBank accession numbers for the examined orchid species.

(An asterisk indicates that chromosome data are reported here for the first time, to our knowledge.)

species	chromosome number ($2n$)	A1 (intrachromosomal asymmetry index) (± 0.01)	GenBank accession numbers (ITS1 and ITS2)
<i>Anacamptis pyramidalis</i>	36	0.30	Z94061, Z94062
<i>A. collina</i>	36	0.33	Z94075, Z94076
<i>A. coriophora</i>	36	0.42	Z94077, Z94078
<i>A. laxiflora</i>	36	0.24	Z94083, Z94084
<i>A. morio</i>	36	0.21	Z94091, Z94092
<i>A. palustris</i>	36	0.33	Z94093, Z94094
<i>A. papilionacea</i>	32	0.38	Z94095, Z94096
<i>Dactylorhiza romana</i>	40	0.30	AJ606131, AJ606132
<i>D. saccifera</i>	40	0.27	Z94065, Z94066
<i>Gymnadenia conopsea</i> *	40	0.44	Z94067, Z94068
<i>G. nigra</i> *	40	0.39	AJ606129, AJ606130
<i>Himantoglossum hircinum</i>	36	0.22	Z94071, Z94072
<i>H. robertianum</i>	36	0.24	Z94063, Z94064
<i>Neotinea lactea</i>	42	0.34	Z94081, Z94082
<i>N. tridentata</i>	42	0.33	Z94113, Z94114
<i>Ophrys tenthredinifera</i>	36	0.23	Z94111, Z94112
<i>O. fusca</i>	36	0.26	AY014531
<i>Orchis anthropophora</i> *	42	0.39	Z94059, Z94060
<i>O. mascula</i>	42	0.39	Z94087, Z94088
<i>O. provincialis</i>	42	0.44	Z94101, Z94102
<i>O. simia</i> *	42	0.36	Z94107, Z94108
<i>Serapias apulica</i>	36	0.52	AJ606133, AJ606134
<i>S. parviflora</i>	36	0.39	AY014550
<i>S. politisii</i>	36	0.40	AJ606135, AJ606136
<i>S. vomeracea</i>	36	0.46	AY014551

4. DISCUSSION

Our results show that Mediterranean Orchidinae species pairs that share a common pollinator pool have more divergent karyotypes than species pairs that attract different, specific pollinators (figure 1). This effect is not biased by phylogenetic relationships among species pairs, and also is not a consequence of larger genetic distances between species in pairs that share pollinators, compared to pairs with distinct, different pollinators. Also, this effect occurs in all major Orchidinae lineages and is independent of basic chromosome numbers (table 1), which range between $2n = 36$ in *Anacamptis*, *Himantoglossum*, *Serapias* and *Ophrys*, and $2n = 42$ in *Orchis* and *Neotinea* (Pridgeon *et al.* 1997; D'Emerico 2001).

This result sheds new light on the importance of postzygotic isolation mechanisms in orchids with unspecific pollination, and on the role of pollinator specificity for species with specific pollinators.

In closely related Orchidinae that share a pool of pollinators, grow in sympatry and have overlapping flowering periods, karyotype differences may act as postzygotic reproductive isolation mechanisms, and thus may play an important role in the maintenance of species boundaries in sympatry. The karyotype asymmetry index is a good expression of the general morphology of plant chromosomes and the magnitude of its difference is an indirect indication of the number of chromosome rearrangements that have occurred between species. Because the frequency of chromosomal rearrangements affects chromosome pairing in hybrid individuals, the karyotype asymmetry index difference estimates hybrid fertility (Stebbins 1971).

Karyotype differences and pollinator changes may both evolve among geographically isolated populations, leading to the formation of new species in the case of allopatric speciation. Chromosomal rearrangements become fixed rapidly in small, isolated populations through random genetic drift and inbreeding (Levin 2002). When formerly allopatric species come into secondary contact, they may either hybridize and eventually merge into a single species or maintain species differences due to reproductive isolation. In species pairs with different, specific pollinators, there is no need for strong postzygotic isolation, whereas in species pairs that share a common pool of pollinators, species differences can be maintained only if sufficient postzygotic reproductive isolation mechanisms have evolved in allopatry. Based on this scenario, we suggest that the over-representation of chromosomal differences in species pairs that share pollinators represents a kind of reproductive isolate selection (Rieseberg 2001) in which only strongly karyotypic divergent species survive the challenge of sympatry.

While the evolution of karyotype variation in allopatric populations or species seems the most probable scenario, we cannot rule out that chromosomal rearrangements may have occurred in sympatric conditions and have led to the evolution of reproductively isolated taxa without changes in floral morphology and pollinator specificity. Chromosomal rearrangements would then be the mechanism of speciation (White 1978) and not only a postzygotic isolation mechanism that evolved as a by-product of allopatric divergence. The newly formed species may then have retained their ancestral pollinators, which they now share.

It would be of interest to carry out similar analyses in other orchid lineages, but karyotype information is often lacking as a consequence of the difficulties associated with the analysis of chromosome morphology in orchids. However, in the phylogenetically distant European genus *Epipactis* (tribe Neottieae), a large variation in chromosomal asymmetry has been found among closely related sympatric species that attract the same pollinators with their nectar rewarding flowers (D'Emérico *et al.* 1999).

Chromosomal fusions have been indicated to represent synapomorphies for major clades within the subtribe Orchidinae (Bateman *et al.* 2003) but with the exception of some polyploid species, chromosomal rearrangements leading to changes in chromosome numbers are very rare in terminal clades of European Orchidinae (D'Emérico 2001). By contrast, however, available Giemsa C-banding data for *Serapias* and *Orchis* indicate that structural rearrangements in the chromosome complement may be more widespread and are mainly due to the presence of large heterochromatin bands that are often located at centromeric positions and may be resulting from the amplification of repetitive DNA regions (D'Emérico 2001; D'Emérico *et al.* 2002a).

The question that remains is whether karyotype changes occur at a high enough rate for them to play an important role in the evolution of reproductive barriers in Mediterranean orchids? Preliminary karyological data of insular (D'Emérico *et al.* 2002a) or peripheral segregates of otherwise widespread taxa (D'Emérico *et al.* 2002b) indicate the frequent occurrence of small chromosomal changes in the insular or peripheral taxa, compared to the widespread taxon. Also, taking into consideration the high level of geographical fragmentation in the Mediterranean area, and the fact that chromosomal changes become fixed most easily in small and isolated populations such as islands, we suggest that chromosomal differences evolve frequently enough to play an important role in Mediterranean orchid diversification.

While our results highlight the importance of post-zygotic isolation mechanisms in groups that have non-specific pollination, they also support a prominent role for pollinator specificity in groups with specific pollinators, such as in sexually deceptive *Ophrys*. The absence of post-zygotic isolation mechanisms in the latter group suggests that pollinator specificity is indeed important for the evolution and maintenance of species boundaries (Schiestl *et al.* 1999), even if molecular evidence suggests that gene flow across species boundaries may occur (Soliva & Widmer 2003).

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