Genetic patterns and pollination in *Ophrys iricolor* and *O. mesaritica* (Orchidaceae): sympatric evolution by pollinator shift

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Ophrys iricolor and *O. mesaritica* are a pair of morphologically similar, closely related sexually deceptive orchids from the eastern Mediterranean. *Ophrys iricolor* is known to be pollinated by *Andrena morio* males and the specific pollinator of *Ophrys mesaritica* is determined as *Andrena nigroaenea*. Amplified fragment length polymorphism revealed *O. iricolor* and *O. mesaritica* to be genetically intermixed on the whole, although populations of *O. iricolor* and *O. mesaritica* in geographical proximity are strongly differentiated, suggesting that specific pollinators locally differentiate these taxa. Based on the available biological data and the system of pollinator attraction operative in *Ophrys*, we hypothesize that *O. mesaritica* may have arisen from *O. iricolor* by pollinator shift and that this is more probable than scenarios invoking hybridization as a result of mispollination by rare, non-specific flower visitors or specifically attracted insects. © 2009 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2009, **159**, 583–598.

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INTRODUCTION

Verne Grant coined the concept of floral isolation among plant species, which describes the combination of ethological and mechanical prezygotic reproductive isolation acting at the stage of pollination (Grant, 1949). Ethological isolation results from separate pollinators exhibiting distinct behavioural responses to flowers of different plant species, whereas mechanical isolation can come about if the pollen of separate plant species is carried on different parts of the same pollinator. Floral isolation (also called pollinator isolation) and pollinator shifts are well documented from a number of different plants, including *Aquilegia* L. (e.g. Grant, 1952; Hodges & Arnold, 1994; Fulton & Hodges, 1999; Hodges *et al.*, 2003; Whittall & Hodges, 2007), *Mimulus* L. (e.g. Bradshaw *et al.*, 1995, 1998; Schemske & Bradshaw, 1999; Bradshaw & Schemske, 2003) and various members of Polemoniaceae (Grant & Grant, 1965). Floral isolation is also common in Orchidaceae (reviewed in Schiestl & Schlüter, 2009), being found for instance in *Anacamptis* Rich. (e.g. Dafni & Ivri, 1979), *Bulbophyllum* Thouars (Borba & Semir, 1998), *Chiloglottis* R. Br. (Bower, 1996), *Cypripedium* L. (Bänziger, Sun & Luo, 2008), *Disa* Berg. (e.g. Johnson & Steiner, 1997), *Ophrys* L. (e.g. Kullenberg, 1961; Paulus & Gack, 1990a; Schlüter *et al.*, 2007b) and *Satyrium* Sw. (e.g. Johnson, 1997).

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Particularly striking in the orchid family is the use of deception for pollinator attraction and floral isolation (Dafni, 1984). In food deception, orchid flowers imitate signals present in flowers of other plants that are used by pollinators to identify these as food plants, whereas, in sexual deception, orchid flowers imitate sexual signals of female insects used for the attraction of conspecific males (Dafni, 1984). The signals employed in sexual deception elicit pollinator reproductive behaviour and have been reported to be highly insect species-specific, thereby allowing for strong ethological isolation (Paulus & Gack, 1990a). Because of their high specificity, sexually deceptive systems are typically characterized by strong prezygotic isolation and weak post-mating barriers and, in connection with this, the potential for the fast evolution of reproductively isolated populations and taxa (Cozzolino, D'Emerico & Widmer, 2004; Cozzolino & Widmer, 2005; Scopece *et al.*, 2007). Differential attractiveness of flowers to pollinators can lead to the build-up of floral isolation, and speciation by a shift in predominant pollinator(s) and pollinator-mediated selection, creating two reproductively isolated sister species differing in their pollinators (Grant, 1949; Paulus & Gack, 1990a; Grant, 1994; Waser & Campbell, 2004).

The genus *Ophrys* provides good examples of floral isolation, exhibiting both mechanical and ethological isolation mechanisms. Different species of *Ophrys* can be in mechanical isolation as a result of differential placement of pollinaria on their pollinators. For example, *O. sphegodes* Miller attaches its pollinaria to the head of the mining bee *Andrena nigroaenea*, whereas *O. lupercalis* Devillers-Terschuren & Devillers attaches them to the abdomen of the same bee species (Paulus & Gack, 1990a). The orientation of the pollinator on the orchid flower, and consequently the body part with which it removes pollinaria, is determined by trichomes on the labellum surface (Ågren, Kullenberg & Sensenbaugh, 1984; Pirstinger, 1996; Schlüter & Schiestl, 2008).

Ethological isolation in *Ophrys* is conveyed by a system of sexual deception that is based upon the chemical mimicry of the sex pheromone and, to a lesser extent, visual and tactile characters, of virgin female pollinators (Kullenberg, 1961; Paulus & Gack, 1990a; Schiestl *et al.*, 1999, 2000; Paulus, 2006). *Ophrys* flowers mimic sex pheromones that are blends of multiple chemicals, mostly hydrocarbons (alkanes and alkenes) of different chain lengths and double-bond positions (Schiestl *et al.*, 1999, 2000; Schlüter & Schiestl, 2008). These hydrocarbons are generally counted among the components of the cuticular wax layer (Schiestl *et al.*, 2000, and references therein) and are expected to be products of a very-long-chain fatty acid (VLCFA) synthesis, a ubiquitous biochemi-

cal pathway (Harwood, 1997; Rawlings, 1998; Millar, Smith & Kunst, 2000; Kunst & Samuels, 2003; Kunst, Samuels & Jetter, 2005; Schlüter & Schiestl, 2008). The presence of alkenes in the orchid subtribe Orchidinae may have served as a pre-adaptation for the evolution of sexual deception in *Ophrys* (Schiestl & Cozzolino, 2008). The combinatorial nature of the sex pheromone implies that a change in sex pheromone, and thus pollinator specificity, may be because of a small number of genetic changes. For instance, a different pattern of alkanes and alkenes may easily be produced by a change in substrate specificity or regiospecificity (the positioning of double-bond insertion) in a fatty acid (acyl-CoA or acyl-ACP) desaturase or acyl-CoA elongase, or simply reflect a change in expression level in any of components of the VLCFA synthesis machinery (see e.g. Shanklin & Cahoon, 1998; Todd, Post-Beittenmiller & Jaworski, 1999; Hildebrand *et al.*, 2005; Schlüter & Schiestl, 2008).

As a consequence of sex pheromone mimicry, *Ophrys* pollination is typically highly specific, with one or few pollinators per *Ophrys* species (Paulus & Gack, 1990a; Paulus, 2006). Speciation may be linked to a pollinator shift (Paulus & Gack, 1990a; Schiestl & Ayasse, 2002; Schlüter & Schiestl, 2008) and, as such a shift may occur within a single population, it is conceivable that speciation can occur on a relatively short timescale, in sympatry, or as a progenitor-derivative speciation event (see e.g. Witter, 1990; Levin, 1993; Rieseberg & Brouillet, 1994; Perron *et al.*, 2000). Given sufficient time for neutral genetic variation to accumulate, one would expect that populations reproductively isolated by different pollinators should be genetically differentiated, a situation which has been recently been documented among some members of the *O. omegaifera* Fleischmann *s.l.* species group (Schlüter *et al.*, 2007b). Nonetheless, in spite of high pollinator specificity, species-specific pollination is not absolute and this leakiness in ethological, and sometimes even mechanical species barriers can lead to hybridization and gene flow among *Ophrys* species, which can be difficult to distinguish from ancestral polymorphism accompanying recent speciation (e.g. Mant, Peakall & Schiestl, 2005; Schlüter *et al.*, 2007b; Devey *et al.*, 2008; Stökl *et al.*, 2008; Cortis *et al.*, in press; Vereecken, 2009; this study).

Here, we investigate pollination and speciation in *O. iricolor* Desf. and *O. mesaritica* H. F. Paulus, C. Alibertis & A. Alibertis, that, based on morphology, amplified fragment length polymorphism (AFLP) and *LFY* sequence data (Schlüter *et al.*, 2007a and P. M. Schlüter *et al.*, unpubl. data), are closely related and likely sister species. The recent systematic treatment of Pedersen & Faurholdt (2007) includes *O. mesaritica* under *O. fusca* Link subsp. *fusca* and refers to *O. iricolor* as *O. fusca* subsp. *iricolor* (Desf.)
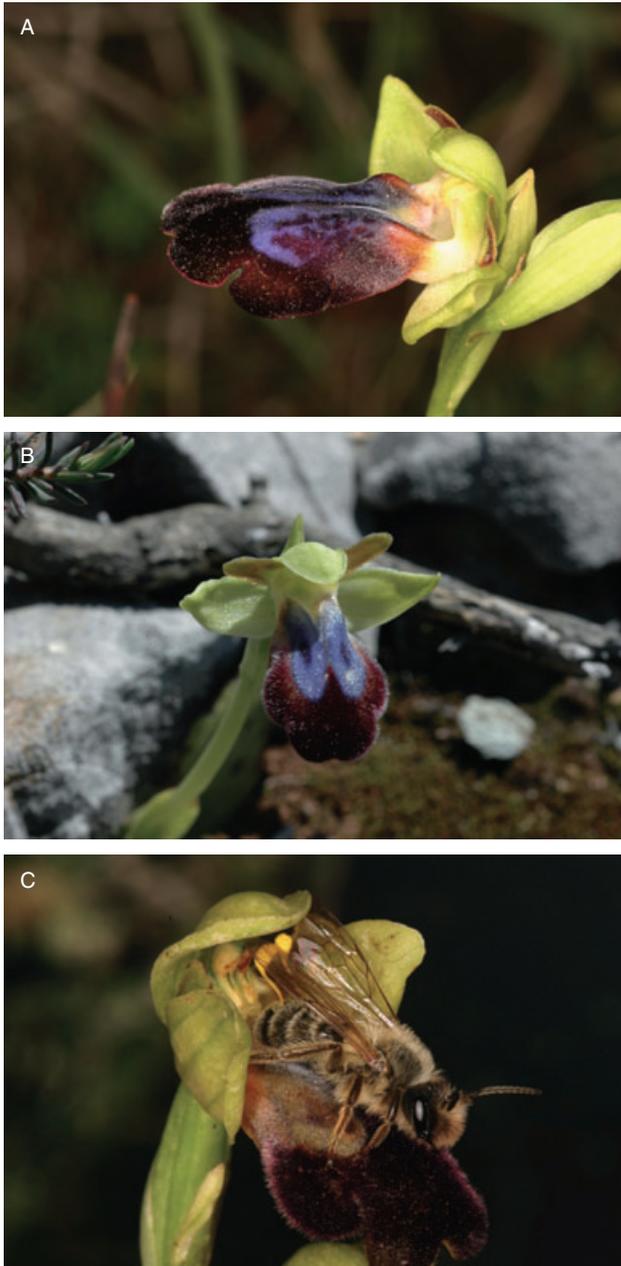
Figure 1. A–C, photographs of flowers of the studied taxa. A, *Ophrys iricolor* (Jouchtas, Crete, 27 April 2004). B, *O. mesaritica* (Ag. Pelagia, Kythira, 17 March 2005). C, *Andrena nigroaenea* pseudocopulating with an *O. mesaritica* flower (17 March 2005, Orino, Crete, plant from Zaros, Crete). Photos (A) and (C) by HFP, (B) by PMS.

K. Richt., but we will here refer to them at the species rank (as in Delforge, 2005) and note that species delimitation in *Ophrys* is a debated issue (for different views, see e.g. Paulus & Gack, 1990a; Delforge, 2005; Pedersen & Faurholdt, 2007; Devey *et al.*, 2008). *Ophrys iricolor* and *O. mesaritica* (Fig. 1) are

members of the monophyletic *Ophrys* section *Pseudophrys* (e.g. Soliva, Kocyan & Widmer, 2001; Bateman *et al.*, 2003), members of which attach pollinaria to the abdomen rather than the head of a pollinator (Kullenberg, 1961; Paulus & Gack, 1994). The vast majority of species in this section are *Andrena*-pollinated members of the *O. fusca s.l.* complex. However, *O. iricolor* and *O. mesaritica* can be separated from this complex relatively easily using morphological characters (Paulus, Alibertis & Alibertis, 1990; Paulus, 1998). Among species of section *Pseudophrys* in the eastern Mediterranean, only *O. mesaritica* bears a strong resemblance to *O. iricolor* and these two could represent a progenitor-derivative species pair. They differ in floral characters such as the size of the lip and in phenology, distribution and pollinators (Paulus, 1998). *Ophrys iricolor* has relatively large flowers that are pollinated by *Andrena morio* (Hymenoptera: Andrenidae), is distributed in the east Mediterranean and is common in the Aegean. *Ophrys mesaritica*, which flowers earlier and has smaller flowers, was previously thought to be restricted to southern Crete and to have a different pollinator, although the identity of the pollinator remained elusive (Paulus *et al.*, 1990). *Ophrys iricolor* accessions from Sardinia, like most *Ophrys*, have been shown to be diploids (D'Emerico *et al.*, 2005), but there are no chromosome counts available from any Aegean populations.

To investigate the origin of *O. mesaritica*, it is necessary to understand the relationship of *O. mesaritica* to *O. iricolor*. Therefore, we used AFLP molecular markers (Vos *et al.*, 1995) to investigate the genetic structure of *Ophrys* populations. AFLP are dominant, quasi-neutral markers that have been shown to be useful in studies of plant populations and closely related species and have been applied to questions of population structure, relationship, speciation and genetic diversity (e.g. Hedrén, Fay & Chase, 2001; Beardsley, Yen & Olmstead, 2003; Marhold *et al.*, 2004; Schönswetter *et al.*, 2004; Tremetsberger *et al.*, 2004; Pfosser *et al.*, 2006; Savolainen *et al.*, 2006).

The present paper aims to elucidate the origin and evolution of *O. mesaritica*, addressing the hypothesis that *O. mesaritica* and *O. iricolor* could be a sister-species pair that originated by a shift in pollinator. This is carried out by investigating the pollination biology of *O. mesaritica* in comparison with *O. iricolor* and analysing the genetic differentiation and relationships of groups with different pollinators.

MATERIAL AND METHODS

POLLINATOR TESTS

As pollinator visits are rare and bees in a given location may already have learned to avoid local

Ophrys individuals, pollinator tests were carried out in the field (see e.g. Paulus, Gack & Maddocks, 1983; Paulus & Gack, 1984; Paulus, 2006). Plants (stalks with open flowers) were picked and taken to areas where male bees were patrolling to test whether flowers would be attractive to the bees. Copulation attempts and approaches by bees to the flowers were noted and documented photographically wherever possible. Successful removal of pollinaria was checked and bees caught for identification. Multiple flowers were tested on various bee taxa whenever feasible. In addition, choice experiments were carried out, in which male bees were presented with *Ophrys* individuals from different taxa, or to a bouquet of *Ophrys* flowers, to investigate which flower (if any) a given bee would choose. The taxonomic identity of bees was kindly ascertained by Fritz Gusenleitner (Biocentre Linz, Austria).

Additional choice tests (termed 'acceptance tests') were carried out on captured male bees, presenting two flowers of putatively closely related *Ophrys* taxa to a bee in a container. These flowers alternated between experiments and consisted of a control flower, expected to be ineffective in eliciting pseudocopulatory behaviour and a second flower expected to be attractive. In this test, male bees will typically crawl over flowers that are uninteresting to them, but only attempt to copulate with flowers that are attractive to them. A sexual response is evident if males immediately start copulatory behaviour, position themselves correctly on the flowers, buzz their wings, commence intense copulatory movements and remove pollinaria (if still present).

PLANT MATERIAL FOR MOLECULAR ANALYSES

Plant material (Table 1 and Fig. 2) was collected in the field and leaf material stored in silica gel. Wherever possible, photographs of all plant individuals were taken and representative vouchers deposited in the herbarium of the University of Vienna (WU), Austria and the herbarium of the Balkan Botanic Garden in Kroussia, Greece. In part as a result of the unexpected results of the pollinator tests, a second sample (data set 2) was collected in 2005.

Plant material from *O. iricolor* subsp. *maxima* (Terracciano) Paulus & Gack was available for comparison with the first data set. This material was collected by HFP in Malta on 30 December 2003 (Dingli Cliffs, $N = 5$, sample 158) and 31 December 2003 (Wardja Ridge, $N = 4$, sample 160).

DNA EXTRACTION, AFLP REACTIONS AND SCORING

DNA was extracted using a DNEasy plant mini kit (Qiagen), following the manufacturer's protocols.

AFLP reactions were carried out following the protocol of Vos *et al.* (1995), with minor modifications (Schlüter *et al.*, 2007b), including positive and negative controls. AFLPs with fluorescence-labelled *Eco*RI primers used the following primer combinations: *Mse*I-CTCG with *Eco*RI-ACT (6-FAM), -ATC (HEX), -ACC (NED) and *Mse*I-CTAG with *Eco*RI-ACT (6-FAM), -AGG (HEX), -AGC (NED). Fluorescence was recorded on an ABI Prism 377 DNA sequencer (Perkin Elmer). All data were scored manually twice, using Genographer software v.1.6.0 (Benham *et al.*, 1999) as a visual aid, and coding bands as presence (1) or absence (0), explicitly scoring ambiguous bands as missing data (?).

DATA ANALYSIS

AFLP data were used for dendrogram construction. Nei & Li's (1979) distances were calculated in PAUP*4.0b10 (Swofford, 2002) and subjected to clustering by the unweighted pair group method using arithmetic averages (UPGMA) and neighbor joining (NJ) (Sokal & Sneath, 1963; Saitou & Nei, 1987). Standard Jaccard and Dice similarity (Jaccard, 1908; Dice, 1945; Sørensen, 1948) and, to take into account the potential effect of missing data, the minimum, maximum and average Jaccard and Dice similarities (Schlüter & Harris, 2006) were calculated using FAMD 1.1 (Schlüter & Harris, 2006; available from www.famd.me.uk) and clustered using UPGMA or NJ in FAMD or PAUP*, respectively. Bootstrap analysis with 1000 pseudo-replicates was done with the same software. Principal coordinate analysis (PCoA; Gower, 1966) was performed on a normal and average Jaccard distance matrix in SYNTAX 2000 (Podani, 2001) and FAMD. Bayesian model-based clustering of individuals was carried out using BAPS 3.2 (Corander, Waldmann & Sillanpää, 2003), treating AFLP data as diploid and coding the second allele at every locus as missing data. Runs were performed with the maximum number of populations set to 5 or 10. Similar analyses were performed including *O. iricolor* subsp. *maxima*.

Analysis of molecular variance (AMOVA; Excoffier, Smouse & Quattro, 1992) was performed on the AFLP data using Arlequin 3.0 (Excoffier, Laval & Schneider, 2005), which calculates the Euclidean distance and discounts loci with more than 5% missing data. AMOVA analyses in Arlequin were performed across all loci and on a locus-by-locus basis. To check for potential effects of missing data, these AMOVA values were compared against values (across all loci) using an average Jaccard's coefficient and Euclidean and Jaccard coefficients calculated in FAMD, either ignoring missing data or randomly replacing them by 50% band presences.

Table 1. Accessions used in the present study

Island/region	Symbol	Locality	Code	Date*	N	Data set	Collector	Sample number
Ophrys iricolor Desf. (N = 24)‡								
Crete	●	Agies Paraskies	APA	30 March 2003	4	1	HFP	106
Greece: Attica	▼	Athens	ATH	26 March 2004	2	1	M. Fiedler	208
Samos	◀	Palaeokastro	EPK	26 February 2004	2	1	HFP	187
Kos	▶	Kephalos	KEF	2 March 2002	1	1	HFP	068
Rhodes	+	Kolymbiae	KOL	27 March 2004	2	1	M. Fiedler	213
Samos	◀	Ormos	SWO	24 February 2004	1	1	HFP	183
Kephallonia	▲	Argostoli	AGS	27 March 2005	5	2	HFP	337
Crete	●	Kalivitis, S of Orino	KVT	20 March 2005	5	2	HFP	309
Karpathos	×	Piles	PIL	23 March 2005	2	2	PMS	318†
O. mesaritica H. F. Paulus, C. Alibertis & A. Alibertis (N = 41)§								
Crete	●	Phaistos	FES	20 February 2004	9	1	PMS	161
Crete	●	Miamou	MIA	24 February 2004	6	1	PMS	170
Crete	●	Zaros	ZAR	16 March 2005	5	2	HFP	290
Kythira	■	Ag. Pelagia	APL	17 March 2005	7	2	PMS	330
Kythira	■	Kapsali	KPS	17 March 2005	2	2	PMS	292
Kythira	■	Frillingianka	FRI	18 March 2005	1	2	PMS	298
Kythira	■	Fratsia	FRA	18 March 2005	1	2	PMS	297
Kythira	■	Mitada	MIT	18 March 2005	2	2	PMS	350
Leukas	◆	Levkada	LEV	23 March 2005	4	2	M. Hirth	335
Kephallonia	▲	Fiskardo	FIS	28 March 2005	1	2	HFP	341
Kephallonia	▲	Valsamata	VAL	29 March 2005	3	2	HFP	343

*The vegetation was late in the year 2005; the sampling dates for *O. mesaritica* therefore seem atypically late.
†These plants were partly withered and are therefore treated as *O. cf. iricolor*.
‡These symbols are reproduced in blue in figures 2 and 3.
§These symbols are reproduced in red in figures 2 and 3.
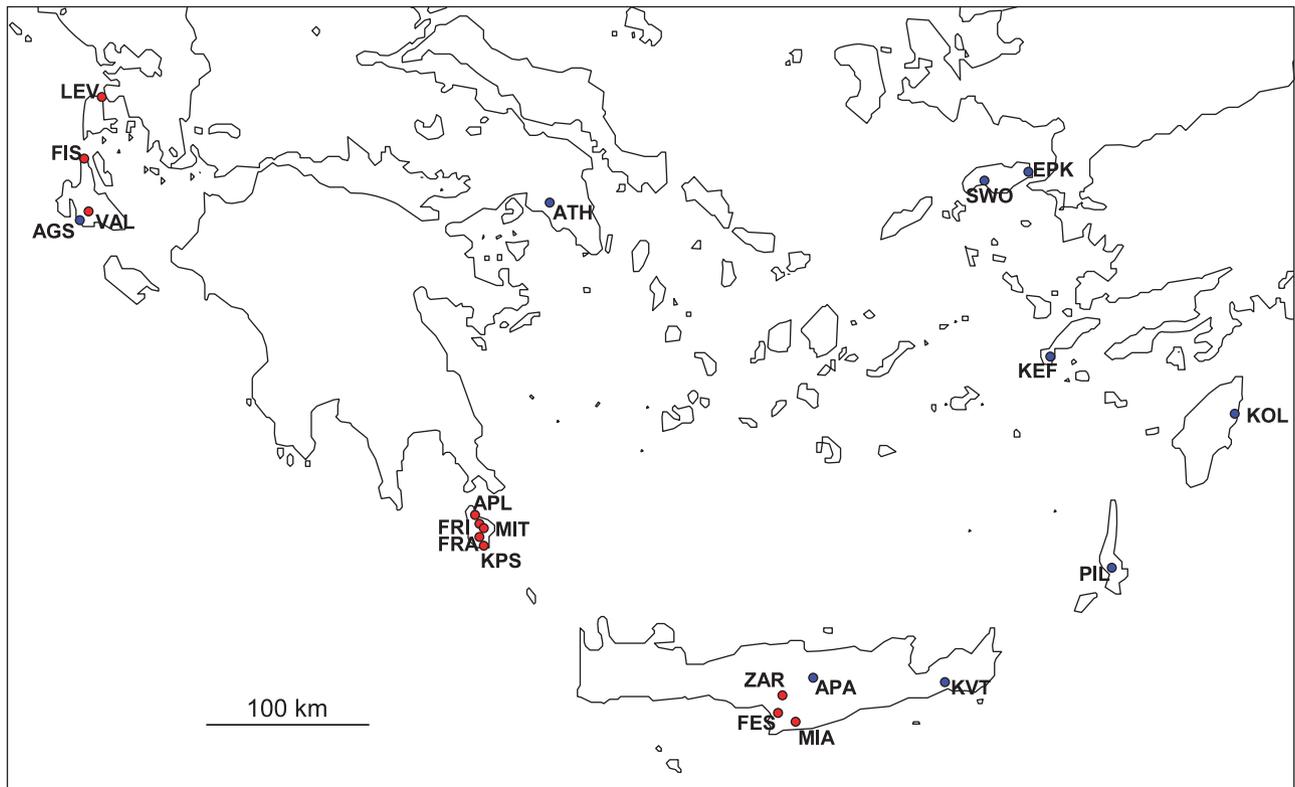N represents the number of individuals sampled from a given locality.

Figure 2. Map of Greece, with population codes indicating sampling localities as given in Table 1. Sampling localities for *Ophrys iricolor* are depicted in blue and for *O. mesaritica* in red.

To test for the correlation of genetic and geographic distances, Mantel tests were performed (Mantel, 1967; Sokal & Rohlf, 1995). Geographic distances were calculated as great circle distances using the haversine formula (Sinnott, 1984) and Microsoft Excel. Two approaches were used to calculate genetic inter-population distances: (1) population allele frequencies were estimated from AFLP data using the Bayesian method of Zhivotovsky (1999) with a uniform prior distribution and these allele frequencies were then used to calculate the chord distance of Cavalli-Sforza & Edwards (1967) in the multi-locus formulation of Takezaki & Nei (1996); and (2) pairwise AMOVA-derived Φ_{ST} values were calculated from AFLP data based on an average Jaccard similarity ($r = 100$, Schlüter & Harris, 2006) and used as a proxy for inter-population genetic distance. Approaches (1) and (2) were implemented and calculated in FAMD 1.1 software. Mantel tests were performed using zt software (Bonnet & van de Peer, 2002) and the exact-permutations procedure. Tests were carried out for data sets 1 and 2, in each case excluding those localities where only a single individual was available. Similar tests, based on the chord distance, were repeated for subsets of data set 2 (groups B and C, see Results and Fig. 3B) separately. Because of the

requirement for data matrices of a size of at least 5×5, one subset of data set 2 (group A) could not be tested. The same limitation also necessitated the inclusion of single-individual 'populations' in the test matrices.

RESULTS

POLLINATOR TESTS

Field observations showed that *O. mesaritica* plants from Crete, Corfu, Kythira and Leukas (Ionian islands) were attractive to *A. nigroaenea* and elicited pseudocopulatory behaviour (Fig. 1C). *Andrena nigroaenea* did not respond to *O. iricolor* flowers. Observations of pseudocopulation were made in Crete and Leukas, in March 2005. These data are consistent with earlier observations (H. F. Paulus, M. Hirth & C. Gack, unpubl. data; Table 2) made near Scourades on Corfu in early March 2000, although the taxonomic identity of the *A. nigroaenea*-pollinated *Ophrys* taxon as *O. mesaritica* was not clear at that time. Pollination of *O. iricolor* by *A. morio* is well documented and *O. iricolor s.s.* does not elicit sexual responses in *A. nigroaenea* (see Paulus & Gack, 1990a, b, c, 1995). *Andrena morio* males do not appear to be present
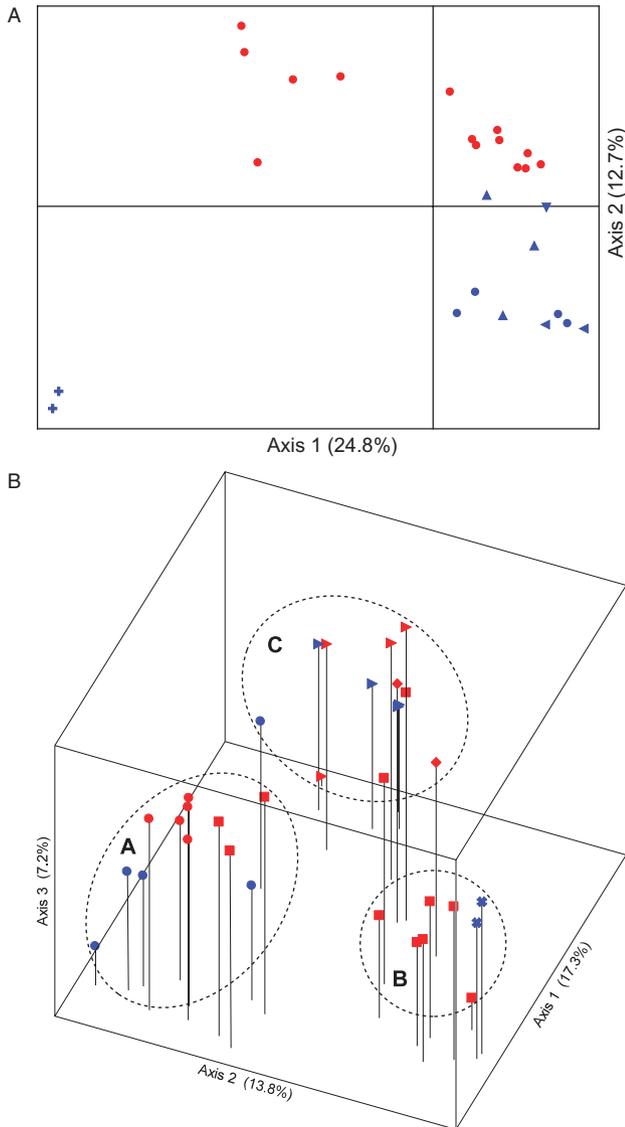
Figure 3. A–B, principal coordinate analysis (PCoA) based on an average Jaccard coefficient ($r = 100$, Schlüter & Harris, 2006). *Ophrys iricolor* and *O. mesaritica* are shown in blue and red, respectively, in both plots. Symbols for sample origin are listed in Table 1. Variation explained by the first PCoA axes is indicated in the plots. A, first data set. B, second data set, where letters (in bold) indicate groups: group A, mainly Crete; group B, mainly Kythira; group C, mainly Kephallonia (for further details, see text).

during the blooming season of *O. mesaritica* on Crete and hence are unavailable as pollinators.

Field choice tests (Table 2), in which bouquets of different *Ophrys* species were presented to *A. nigroaenea* males, resulted in pseudocopulations only on *O. mesaritica* and *O. lupercalis*, the latter already known to be pollinated by that bee, although judging from the behaviour of the bees, *O. mesaritica* appeared slightly more attractive to pollinators than *O. lupercalis*. None of the other *Ophrys* taxa (including *O. iricolor*) presented together with *O. mesaritica* elicited mating behaviour in *A. nigroaenea*.

Acceptance tests (Table 3) on captured *A. nigroaenea* and *A. flavipes* males corroborated the results from field experiments and showed a 100% sexual response of *A. nigroaenea* to *O. mesaritica* flowers under artificial conditions, whereas male bees of the same species completely ignored *O. iricolor* and *O. leucadica* flowers. Conversely, *A. flavipes*, another relatively common pollinator in *Ophrys*, did not exhibit any mating behaviour towards *O. mesaritica* flowers.

AFLP RESULTS

Data from two scorings of the AFLP data yielded 1037 (519 + 518) AFLP fragments for 27 individuals and 1014 (507 + 507) fragments for 38 individuals for the samples collected prior to 2005 (data set 1) and in 2005 (data set 2), with 3.97 and 7.23% missing data present in these data sets. The second data set contained five individuals for which data from one primer combination could not be retrieved because of technical problems. However, preliminary analysis suggested that the data obtained for these individuals did not preclude genetic assignment to the expected sample groups; the necessary care was taken in the analysis of these individuals. After removal of bands only occurring in single individuals, 825 (data set 1) and 847 (data set 2) AFLP bands were available. Both scorings of each of the AFLP data matrices yielded congruent results during initial analyses and were therefore combined subsequently.

Principal coordinate analyses (Fig. 3A) of the first (pre-2005) data set showed *O. mesaritica* and *O. iricolor* separated along the second axis of variation (12.7%), whereas the Bayesian method assigned groups along the first axis of variation (24.8%). Dendrograms showed an intermediate picture, bootstrap support for different groupings being generally low (<70%). In the strict consensus, only the isolated position of two *O. iricolor* samples from Rhodes was confirmed. When Maltese *O. iricolor* subsp. *maxima* were included in the analysis, they appeared as a distinct cluster in PCoA and dendrograms, typically with 100% bootstrap support (Fig. 4), whereas *O. mesaritica* clustered only with *O. iricolor*. On inclusion of additional taxa from the *O. fusca s.l.* or *O. omegaifera s.l.* groups, other taxa often occupied positions between *O. iricolor*/*O.mesaritica* and Maltese *O. iricolor* subsp. *maxima*, a pattern which was also observed in *LFY* sequence data (P. M. Schlüter, G. Kohl, T. F. Stuessy and H. F. Paulus, unpubl. data).

The PCoA of the second (2005) data set (Fig. 3B) revealed *O. iricolor* and *O. mesaritica* to be more or

Table 2. Field choice tests of *Andrena nigroaenea* males on sets of *Ophrys* inflorescences (including negative controls), recording the number (*N*) of males pseudocopulating with flowers

Place, date	*Ophrys* species	*N* tested individuals	*N* pseudocopulations*	Plant source	Control plants
Corfu, March 2000†	*O. mesaritica*	20	*c.* 30	Corfu	*O. leucadica*
	O. lupercalis	4	4	Corfu	
Orino, Crete, 15/16 March 2005	*O. mesaritica*	7	8	Zaros, Crete	*O. creticola* *O. creberrima*‡
Ag. Nikolaos, Crete, 25 March 2005	*O. mesaritica*	2	3	Kythira	*O. thriptiensis*
	O. cf. *iricolor*	1	0	Karpathos	*O. fleischmannii*

*As not all bee males could be caught and/or carried pollinia, it is not possible to exclude multiple visitation by the same individuals.
†H. F. Paulus, M. Hirth & C. Gack, unpubl. data.
‡Pseudocopulations with *Andrena creberrima*.

Table 3. Acceptance tests (conducted 28–29 March 2005 and 3 March 2008), with 10 experimental replicates for each bee/plant combination, showing the percentage of plants of a given species that elicited a pseudocopulatory response in the *Andrena* males tested

Ophrys species	*N*	*A. nigroaenea* (*N* = 3 males*)	*A. flavipes* (*N* = 8 males†)	Plant origin	Collection date
O. mesaritica	10	100%	0%	Leukas	26 March 2005
O. mesaritica	2	100%	0%	Kephallonia	28 March 2005
O. iricolor	2	0%	0%	Attica	26 March 2005
O. iricolor	9	0%	0%	Kephallonia	27 March 2005
O. cf. *iricolor*	3	0%	0%	Karpathos	22 March 2005
O. leucadica	7	0%	100%	Attica	26 March 2005
O. leucadica	12	0%	100%	Kephallonia	28 March 2005
O. cinereophila	12	0%	0%	Attica	26 March 2005
Ophrys species	*N*	*A. morio* (*N* = 9 males‡)	*A. flavipes* (*N* = 10 males‡)	Plant origin	Plant date
O. iricolor	9	100%	0%	Cyprus	2 March 2008

A. nigroaenea males from Attica.
†*A. flavipes* males from Attica and Kephallonia.
‡*A. flavipes* and *A. morio* males from Cyprus.
N indicates the number of individuals tested.

less intermingled. Three groups could be identified, which corresponded to groupings found by Bayesian clustering. These groups were (group A) a cluster consisting of *O. iricolor* and *O. mesaritica* from Crete and three *O. mesaritica* individuals from Kythira; (group B) *O. mesaritica* from Kythira and two *O.* cf. *iricolor* samples from Karpathos; (group C) a mix of *O. iricolor* and *O. mesaritica* from the northern islands of Kephallonia and Leukas and a single *O. iricolor* individual from Crete. Dendrograms roughly agreed with this separation of groups and parts of

group C (*O. mesaritica* individuals from populations VAL and LEU) were supported with 74% in a Jaccard/UPGMA bootstrap analysis. Support for group B was <70% and group A was supported with 90%. Within this group, all but one *O. iricolor* individuals were grouped with 82% support, *O. mesaritica* with 99%. Within *O. mesaritica*, samples from Crete and Kythira had 70 and 87% support, respectively.

AMOVA was conducted using different hypotheses of population structure. AMOVA-derived Φ_{ST} values from locus-by-locus calculations (all $P < 0.001$) and
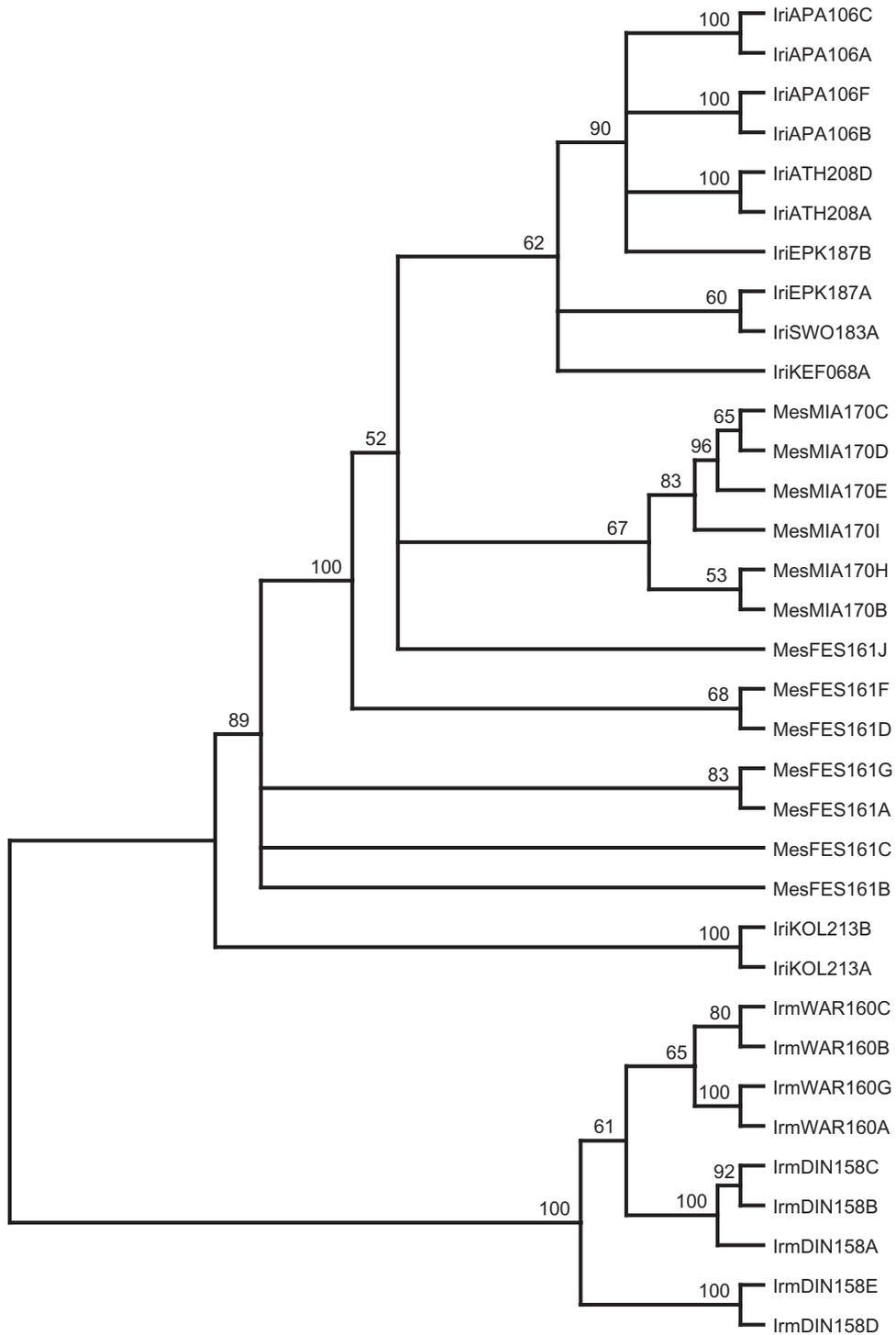
Figure 4. Bootstrap consensus from 1000 neighbour joining (NJ) dendrograms constructed using average Jaccard coefficients ($r = 100$, Schlüter & Harris, 2006), showing bootstrap support above the branches. Labels indicate taxon assignment, where 'Iri', 'Mes' and 'Irm' represent *Ophrys iricolor*, *O. mesaritica* and *O. iricolor* subsp. *maxima*, respectively, followed by locality and sample codes, as given in Table 1, and a letter identifying every plant individual.

Table 4. AMOVA-derived Φ_{ST} values under different hypotheses of population structure

Data set	1	1	2	2	2A	2A	2C	2C
Localities	All	Crete	All	All	All	Crete	All	Ionian islands
Structure	I/M	I/M	I/M	A-C	I/M	I/M	I/M	I/M
$\Phi_{ST,a}$	0.101	0.191	0.022	0.238	0.201	0.196	0.075	0.202
$\Phi_{ST,b}$	0.128	0.231	0.019	0.297	0.275	0.256	0.094	0.244

Population structures tested for different data sets were *Ophrys iricolor* vs. *O. mesaritica* (I/M), with all individuals or only those from the indicated geographic areas (see 'Localities') or structure according to the groups A–C determined during previous analysis (for details see text). $\Phi_{ST,a}$ and $\Phi_{ST,b}$ denote the mean Φ_{ST} across all loci determined by a locus-by-locus calculation (Arlequin software, Euclidean distance) and the Φ_{ST} based on an average Jaccard index (FAMD software; $r = 100$), respectively.

based on average Jaccard's coefficient are shown in Table 4. Φ_{ST} values using different similarity coefficients and after different treatments of missing data were all in agreement and significantly correlated (all $r > 0.95$ and $P < 10^{-4}$). Φ_{ST} values were calculated for *O. iricolor* vs. *O. mesaritica* in the first data set and groups A and C of the second data set. These calculations were performed (1) using all plant individuals assigned to these groups and (2) only using individuals at a given location, i.e. Crete or Kephallonia and Leukas, where one individual from locality FIS was excluded as an outlier. Using (1) all plants assigned to groups, Φ_{ST} values were generally low, whereas (2) in any particular location, Φ_{ST} differentiation was comparatively high and comparable in magnitude to values of other, distinct *Ophrys* section *Pseudophrys* species pairs (e.g. *O. cinereophila* Paulus & Gack vs. *O. leucadica* Renz with $\Phi_{ST,a} = 0.198$; P. M. Schlüter, unpubl. data).

Mantel tests revealed no correlation among genetic and geographic distances in the first data set ($r = 0.210$, $P = 0.268$ using chord distance and $r = 0.240$, $P = 0.271$ using pairwise Φ_{ST}), whereas AMOVA-derived Φ_{ST} values were correlated with geographic distances in data set 2 ($r = 0.291$, $P = 0.062$ using chord distance; $r = 0.370$, $P = 0.024$ using pairwise Φ_{ST}). Given the PCoA plot of data set 2 (Fig. 3B), this was to be expected and may reflect the occurrence of three distinct groups in this data set. Analysis of these groups separately revealed no correlation among geographic and genetic distances for either group 2B (mainly Kythira, $r = -0.059$, $P = 0.558$) or group 2C (Ionian islands, $r = -0.260$, $P = 0.202$); Mantel tests were not possible for group 2A.

DISCUSSION

POLLINATION OF *O. MESARITICA* AND ITS IMPLICATIONS

To understand speciation in *Ophrys*, knowledge of pollination biology of the species involved is essential.

We report here for the first time that the pollinator of *O. mesaritica* is the mining bee *A. nigroaenea* and that *O. mesaritica* occurs outside Crete. We found *O. mesaritica* individuals on the islands of Kythira, Kephallonia and Leukas which are morphologically virtually indistinguishable from those on Crete, attract the same bee species and elicit pseudocopulatory behaviour in this bee which effectively removed pollinia from *O. mesaritica* plants. Field observations suggest that *A. nigroaenea*-pollinated *O. mesaritica* is probably also distributed on Corfu (H. F. Paulus, pers. observ. in 2000), although no DNA samples for analysis were available from there. It remains to be seen whether the distribution of *O. mesaritica* is continuous between Kythira and Kephallonia.

Andrena nigroaenea is an abundant bee throughout Europe (Gusenleitner & Schwarz, 2002) and is a relatively common pollinator in *Ophrys*. It pollinates *O. sphegodes* and *O. grammica* (B. Willig & E. Willig) Devillers-Terschuren & Devillers via pollinia attached to the head of the bee, whereas *O. lupercalis*, *O. sitiaca* H. F. Paulus, C. Alibertis & A. Alibertis, *O. mesaritica* and possibly *O. arnoldii* Delforge are pollinated via pollinia attached to the abdomen (Paulus & Gack, 1990a; Paulus, 2001; Delforge, 2005). As not all *A. nigroaenea*-pollinated taxa are closely related (cf. Soliva *et al.*, 2001; Bateman *et al.*, 2003), it is clear that pollination by this bee has evolved independently several times (cf. Cortis *et al.*, in press, for a case of convergent pollination by *A. morio*). It is therefore conceivable that the evolution of *O. mesaritica* was likewise linked to one or more independent pollinator shifts to *A. nigroaenea* widely available as a pollinator. Of course, the co-occurrence of *Ophrys* taxa using the same pollinating insect and the absence of postzygotic mating barriers (Ehrendorfer, 1980; Cozzolino *et al.*, 2004) could result in hybridization. However, there is no evidence so far for *O. mesaritica* occurring in sympatry with any other *Ophrys* taxon that attaches pollinia to the abdomen of *A. nigroaenea*.

GENETIC STRUCTURE IN *O. IRICOLOR* AND *O. MESARITICA*

Understanding the genetic structure of *Ophrys* species is important for inferring their evolutionary history. The genetic structure within *O. iricolor* and *O. mesaritica* does not correspond to two separate groups matching these named taxa. Rather, there are at least three geographic groups within *O. iricolor/mesaritica*, with plants from the north of the sampled region (Kephallonia and Leukas) different from the south (mainly Crete). Furthermore, *O. iricolor* from Rhodes occupies an isolated position, as does *O. mesaritica* from Kythira (where *O. iricolor* was not sampled). However, it is notable that, in all the groups composed of both *O. iricolor* and *O. mesaritica*, populations in geographical proximity were also strongly differentiated, suggesting that different pollinators select for their favourite 'false females' on both Crete and Kephallonia. This differentiation is unlikely to be a result of genetic drift because, within the aforementioned groups of populations, there was no indication of isolation by distance, as shown by the lack of correlation among geographic and genetic distance matrices.

Recent or ongoing speciation of *O. iricolor* and *O. mesaritica* could in principle explain the genetic pattern observed, but the distribution of these taxa on Crete and the Ionian islands (Kephallonia and Leukas) does not necessarily suggest a recent, single divergence. The presence of separate *O. mesaritica* and *O. iricolor* populations in both the south Aegean and Ionian islands and the apparent intermixture of *O. iricolor* and *O. mesaritica* overall would therefore suggest multiple origins of either *O. iricolor* or *O. mesaritica* or, alternatively, population differentiation in one of these taxa associated with recent gene flow. Notably, the latter scenario would be consistent with the genic view of speciation which predicts species divergence in the presence of gene flow (Lexer & Widmer, 2008, and references therein). As *O. iricolor* is far more common and widespread, we hypothesize that geographic structure in the AFLP data may be primarily because of structure within *O. iricolor*, the putatively older species. Hence, an origin of *O. mesaritica* from *O. iricolor* is more plausible than the reverse. *Ophrys mesaritica* may have originated via sympatric speciation or hybridization of *O. iricolor* with another *Ophrys* taxon utilizing *A. nigroaenea*, such as *O. lupercalis*. Such hybridization is certainly possible, as evidenced by hybrid swarms on Sardinia (Gölz & Reinhard, 1990; Paulus & Gack, 1995; Stökl *et al.*, 2008), which have been referred to by the names of *O. eleonorae* Devillers-Terschuren & Devillers (Devillers & Devillers-Terschuren, 1994) or *O. iricolor* subsp. *maxima* (Paulus & Gack, 1995).

Similar *O. iricolor* subsp. *maxima* plants from Malta have incidentally been treated as *O. mesaritica* by Delforge (1993). Unlike *O. mesaritica*, however, these putative hybrids are attractive to both *A. morio* and *A. nigroaenea* and seem unconnected with our sampled *O. mesaritica* from AFLP and *LFY* sequence data (see also Stökl *et al.*, 2008).

OPHRYS MESARITICA MAY HAVE ORIGINATED FROM *O. IRICOLOR* BY POLLINATOR SHIFT

The likely evolution of *O. mesaritica* can be deduced from knowledge of population and pollination biology. As pollinators select for plants that are attractive to them, they are expected to be involved in shaping population genetic patterns and driving population divergence. In principle, the genetic pattern found in *O. iricolor* and *O. mesaritica* can be explained by (1) accidental, 'non-specific' hybridization, (2) 'specific' hybridization involving the specific attraction of a pollinator or (3) one or more (convergent) pollinator shift(s) in *O. iricolor* populations and divergence as a result of pollinator-mediated selection. Hybridization requires the presence of both *O. iricolor* and *O. mesaritica* in flower at the same time at the same location and may occur by one of two mechanisms (points 1 and 2):

1. Non-specific hybridization by mispollination, in the sense that no specific, sex pheromone-mediated attraction of a pollinator is involved. Hybridization is then merely a consequence of pollination by insects that do not normally act as pollinators and pollen transfer across species boundaries in sympatric *Ophrys* populations is as a result of chance happenings. Under this scenario, stochasticity in pollinator attraction, morphological and genetic patterns may be expected and, given two sufficiently distinct parental species, a diagnosis of hybridity should be possible based on these biological characters. We currently have no evidence supporting this scenario.

2. Specific hybridization, where a pollinator is attracted by the odour bouquet of more than one sympatric *Ophrys* species which results in gene flow across species boundaries. If *O. mesaritica* originated by hybridization, then specific hybridization would be the more plausible hybridization scenario, simply because two independent origins of a population that specifically attracts *A. nigroaenea* by mispollination among the many species co-occurring with *O. iricolor* would seem exceedingly unlikely. One candidate hybridization partner of *O. iricolor* is the *A. nigroaenea*-pollinated *O. lupercalis*, which forms hybrid swarms with *O. iricolor* subsp. *maxima* on Sar-

dinia. However, unlike *O. mesaritica*, the Sardinian hybrids are attractive to both bee species, *A. nigroaenea* and *A. morio* (Stökl *et al.*, 2008), and *O. mesaritica* visually resembles *O. lupercalis* less than might be expected (see below). Also, the distribution of *O. lupercalis* has only limited overlap with *O. iricolor* in our study area and, although material of *O. lupercalis* was not available for AFLP analysis, nuclear *LFY* sequences from *O. lupercalis* appear distinctly different from either *O. iricolor* or *O. mesaritica* sequences (P. M. Schlüter, unpubl. data). Thus, it seems unlikely that *O. mesaritica* is a hybrid among *O. iricolor* and *O. lupercalis*.

3. Speciation by pollinator shift is possible when one or a few individuals in a population attract a novel pollinator and this pollinator then imposes a disruptive selection pressure leading to divergence of attractive plants from the rest of their original population. This may eventually lead to a change in floral traits and flowering time, such that the original pollinator can no longer be attracted, gene flow ceases and reproductive isolation is attained. In order to document speciation by pollinator shift, it is necessary that (1) the plant species under consideration are sister species, (2) species differ in their pollinators and are reproductively isolated and (3) there is a causal link between pollinator preference and species divergence. (1) Demonstration of sister species status requires a complete sampling of candidate taxa and phylogenetic reconstructions that allow an unambiguous assessment of sister-species status. Morphologically, *O. iricolor* and *O. mesaritica* appear distinct from other species of *Ophrys* section *Pseudophrys* (Delforge, 2005) and data from AFLP and *LFY*, the most highly resolving sequence marker that can be applied to *Ophrys* to date, both indicate that *O. iricolor* and *O. mesaritica* are likely sister taxa (Schlüter *et al.*, 2007a and P. M. Schlüter *et al.*, unpubl. data). (2) Our pollinator experiments and available pollinator data indicate different pollinators for *O. iricolor* and *O. mesaritica*, with a high specificity of pollination and likely reproductive isolation conferred by pollinator behaviour, although we cannot exclude the possibility of different potential pollinators acting at a low frequency. (3) Whereas a causal link between pollinator preference and species divergence cannot be deduced from our data, there also is lack of evidence to the contrary. To date, no obvious candidate factors (such as apparent habitat differences) have been identified that would be expected to prevent gene flow and drive genetic divergence, genetic drift being an unlikely factor for divergence (see above). However, the breakdown of

reproductive isolation among Sardinian populations of *O. iricolor* subsp. *maxima* (= *O. eleonorae* at species rank) and *O. lupercalis* is evidently linked to cross-attraction of the pollinators, *A. nigroaenea* and *A. morio* (Stökl *et al.*, 2008). The fact that no such cross-attraction of pollinators by our study species has yet been observed argues that specific pollinators may be the causes for incipient genetic divergence between *O. iricolor* and *O. mesaritica*. Differences in flowering phenology and odour bouquets (Stökl *et al.*, 2008), or local odour preferences by pollinators (Vereecken, Mant & Schiestl, 2007; Vereecken & Schiestl, 2008) are possible reasons for the difference in pollinator specificity among *Ophrys* populations in Greece (this study) and Sardinia (Stökl *et al.*, 2008).

Speciation by pollinator shift provides a more parsimonious explanation than the competing hypotheses from a theoretical standpoint. An origin of *O. mesaritica* by sympatric speciation is more probable than by specific hybridization in a system such as *Ophrys* where a multi-component sex pheromone determines pollinator specificity. Both sympatric speciation and specific hybridization require the attraction of a novel bee as a pollinator. However, unlike sympatric speciation, specific hybridization requires that this novel bee already be a pollinator of another *Ophrys* species. Given the vast number of bees that may potentially act as pollinators (see e.g. Paulus & Gack, 1990a; Paulus, 2006), it appears comparatively unlikely that a newly acquired pollinator would already be associated with another orchid species, making sympatric speciation the simpler explanation.

Therefore, we hypothesize that *O. mesaritica* arose from *O. iricolor* in a mechanism involving sympatric speciation by pollinator shift from *A. morio* to *A. nigroaenea*. Given the distribution of these taxa and possible geographic structure within *O. iricolor*, *O. mesaritica* may have originated twice independently by convergent pollinator shifts in different *O. iricolor* source populations, assuming that cessation of gene flow among these taxa has been attained. Alternatively, a single pollinator shift and spread of *O. mesaritica* is also consistent with our data under the assumption that gene flow among these taxa is recent or ongoing, as expected for genic species divergence scenarios (Lexer & Widmer, 2008). Based on field observations, it is presently difficult to judge which scenario is more likely. While there is no indication of pollinator cross-attractiveness or overlap of phenologies on Crete, our knowledge of the situation on Kythira and the Ionian islands is based on a limited sample. Additional studies involving a broader sampling, and including *O. lupercalis*, may be able to reject or support some of the above hypotheses. The

sex pheromone of *A. morio* has recently been elucidated (Stökl *et al.*, 2007) and is similar to that of *A. nigroaenea*, but differs in the ratios of alkanes and alkenes of given carbon chain lengths. Analysis of floral odour of *O. mesaritica* and careful analysis of differences to the *O. iricolor* odour bouquet may allow one to speculate on an enzymatic function and, possibly, candidate genes that may be responsible for differences in species-specific pollinator attraction and, potentially, sympatric speciation among *O. iricolor* and *O. mesaritica*.

Finally, the suspected case of *O. mesaritica* arising from *O. iricolor* by speciation involving pollinator shift concurs with morphological observations on these taxa. These morphological features would be difficult to explain if a hybrid origin of *O. mesaritica* were assumed. In particular, the flower coloration of *O. iricolor* resembles that of a flower mimicking a female of the large, black *A. morio*; the dark, black colour of the lip corresponding to the black body colour of the pollinator and the shiny blue central spot on the lip resembling the shiny blue wings of *A. morio* females. In contrast, the *A. nigroaenea*-pollinated *O. lupercalis* has a less shiny, more greyish-blue central spot and a brown lip, resembling the brown body colour of *A. nigroaenea*. *Ophrys mesaritica*, although attracting *A. nigroaenea*, does not share this obvious *A. nigroaenea*-like coloration, which would be unlikely to be selected against if it appeared in a hybrid of *O. lupercalis* and *O. iricolor*. Therefore, *O. mesaritica* morphologically resembles an *A. nigroaenea*-pollinated, somewhat smaller-flowered *O. iricolor* of potentially very recent origin. Taken together, our findings suggest the likely origin of *O. mesaritica* from *O. iricolor* by sympatric pollinator shift from *A. morio* to *A. nigroaenea*.

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