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Scent variation and hybridization cause the displacement of a sexually deceptive orchid species[1]

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In the sexually deceptive orchid genus *Ophrys*, reproductive isolation is based on the specific attraction of males of a single pollinator species, mostly bees, by mimicking the female sex pheromone of this species. Changes in the floral odor can lead to hybridization, introgression, and possibly speciation. We investigated hybrid swarms of *O. lupercalis* and *O. iricolor* on Sardinia using behavioral, electrophysiological (GC-EAD), chemical, morphological, and genetic methods (AFLPs). In behavioral experiments, approximately 20% of the flowers from both species and hybrids were attractive to the "wrong" or both pollinator species. Analysis of the EAD-active hydrocarbons in the floral odor showed an overlap in the two species, whereby hybrid individuals could not be separated from *O. iricolor*. The genetic analysis confirmed the hybridization of the species. Plants of *O. iricolor* and hybrids are genetically indistinguishable and form an *O. iricolor* × *lupercalis* hybrid population. Remaining plants of *O. lupercalis* will possibly be displaced by the *O. iricolor* × *lupercalis* hybrid population in the future. Our study showed that in deceptive orchids, variation in the pollinator attracting cues, in this case, scent, can be the first step for speciation and at the same time cause the displacement of a species.

Key words: AFLP; GC-EAD; hybridization; *Ophrys,* Orchidaceae; pollination; Sardinia; sexual deception.

Pollination by sexual deception is a remarkable mechanism of pollination exclusive to Orchidaceae and has so far been reported from Europe, South Africa, South America, and Australia (Dafni, 1984; Ackerman, 1986; Anders Nilsson, 1992). The sexually deceptive genus *Ophrys,* consisting of more than 200 species, is spread throughout the entire Mediterranean region, but it can also be found in central and northern Europe (Delforge, 2006). *Ophrys* flowers imitate female insects in shape, color, and odor to attract males to the flowers and to release innate sexual behavior in male pollinators (Kullenberg, 1961), whereby pollination takes place. Pollinators are mainly bees (Andrenidae, Anthophoridae, Colletidae, Megachilidae, and Apidae), occasionally wasps (Sphecidae and Scoliidae), and two beetle species (*Phyllopertha* and *Blitopertha*, Scarabaeidae; Kullenberg, 1961, 1973; Borg-Karlson, 1990; Paulus and Gack, 1990a; Paulus, 2006).

For successful attraction of the pollinators, the flower odor, which is identical to the female sex pheromone of the pollinating species, is essential (Schiestl et al., 1999; Ayasse et al., 2003; Ayasse, 2006). A few compounds can be decisive, as is the case in the attraction of *Campsoscolia ciliata* by *O. speculum* (Ayasse et al., 2003). Alternatively, a complex mixture of several compounds, such as produced by *Ophrys* species that attract *Andrena* species, can define pollinator attraction (Kullenberg and Bergström, 1976; Tengö, 1979; Borg-Karlson, 1990; Schiestl et al., 1999, 2000; Stökl et al., 2005, 2007). Sympatrically occurring species of *Andrena* are attracted by different mixtures of the same hydrocarbons, as has been shown for the attraction of *A. nigroaenea* and *A. flavipes* to *O. fusca* (=*O. lupercalis*) and *O. bilunulata*, respectively (Schiestl and Ayasse, 2002). Furthermore, distantly related *Ophrys* species that are pollinated by the same *Andrena* species use identical compounds in very similar compositions for pollinator attraction (Stökl et al., 2005), indicating a convergent evolution of pollinator attracting odors.

Normally, an *Ophrys* species attracts only one pollinator species. The high degree of specialization in pollination serves as the principal reproductive isolation between sympatric *Ophrys* species, which are mostly genetically compatible and crossable (Bergström, 1978; Ehrendorfer, 1980; Paulus and Gack, 1990a; Paulus, 2006). Karyotype differences between *Ophrys* species are also lower than in other Mediterranean orchids (Cozzolino et al., 2004). Despite the selective attraction of pollinators, nonlegitimate pollination does occur, and hybrids can sometimes be found (Stebbins and Ferlan, 1956; Danesch and Danesch, 1972; Danesch et al., 1975; Ehrendorfer, 1980). For several species, a hybridogenic origin has been proposed (Paulus, 1988; Paulus and Gack, 1990b).

Flower visits in deceptive pollination systems are often rare and brief (Ayasse et al., 2000), and negative frequency-dependent selection in response to odor learning in deceptive systems may favor variability of the pollinator-attracting signal within orchid populations (Ayasse et al., 2000; Gigord et al., 2001). Within *O. sphegodes* populations, there is considerable odor variation, that minimizes learned avoidance of the flowers and increases the likelihood that a given pollinator would visit several to many different plants within a population (Ayasse et al., 2000). Odor changes as a result of genetic drift, negative

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frequency dependent selection, or hybridization may be the driving forces for speciation because the attraction of a new pollinator by a mutant or by the hybrids may act as a prezygotic isolation barrier (Paulus and Gack, 1990a). Nonlegitimate pollination and hybridization are more likely in species that attract their pollinators with different mixtures of the same compounds than in species that attract their pollinators with different chemical compounds. A slight shift in the amounts of some compounds could lead to the attraction of a different pollinator. No new compounds have to be produced by the plant. As a result of selection by the new pollinator, such plants may adapt to a new ecological niche, and a new species may evolve (Stebbins and Ferlan, 1956; Paulus and Gack, 1990a).

Pollinator-mediated isolation between *Ophrys* species, however, is not always complete. Recent studies have suggested gene flow across species boundaries in sympatric species of the *O. sphegodes* group (Soliva and Widmer, 2003; Mant et al., 2005). Paulus and Gack (1995) even found hybrids of *O. lupercalis* and *O. iricolor* on Sardinia that were attractive to two different pollinator species. Typical *O. lupercalis* and *O. iricolor*, pollinated by *Andrena nigroaenea* and *A. morio*, respectively, can be found distinct in some populations on Sardinia, but in certain populations plants can be found that have intermediate characters and that have been hypothesized to be hybrids (Paulus and Gack, 1995). Flowers of plants in such populations were attractive to the pollinators of both species. The size and morphology of the flower determines the fit and the position of the pollinator on the flower and is therefore a crucial factor for successful pollination (Kullenberg, 1961). In morphological investigations of the Sardinian populations of *O. lupercalis* and *O. iricolor*, as well as *O. lupercalis* from Majorca and *O. iricolor* from Crete, the Sardinian populations were intermediate between the two others (Gölz and Reinhard, 1990).

Morphological and olfactory flower traits of sexually deceptive orchids underlie a strong selective pressure by the pollinator and pose problems for estimating the relationships between species. Neutral genetic markers, such as microsatellites or AFLPs, are suitable to measure the genetic structure of hybrid populations and make it possible to assess gene flow between species (Soliva and Widmer, 2003; Mant et al., 2005; Moccia et al., 2007).

In this study we investigated the role of the pollinator attracting floral scent of *O. lupercalis* and *O. iricolor* for hybridization and introgression and the consequences for processes of speciation in this genus. Hybrid swarms and the parental species were therefore used for (1) molecular analyses (AFLP) in combination with morphometric measurements to delimit species, (2) chemical analyses (GC) to identify pollinator attracting compounds, and (3) behavioral experiments with single flowers to test their attractiveness to pollinators of both parent species.

MATERIALS AND METHODS

Study species—*Ophrys lupercalis* Devillers-Terschuren & Devillers (=*nigroaenea-fusca*; Paulus, 2001) and *O. iricolor* Desfontaines belong to the *Ophrys fusca–lutea* group, which consists of approximately 60 species within section *Pseudophrys*. Several species within the *fusca–lutea* group are morphologically very similar and therefore difficult to classify (Paulus, 2001; Delforge, 2006). *Ophrys lupercalis* is pollinated by *A. nigroaenea* Kirby 1802 and is widespread in the western and central Mediterranean. On Sardinia it blooms from early March to the middle of May. *Ophrys iricolor* blooms on Sardinia from the end of February until the middle of May, has flowers with a typical red underside, and is pollinated by *Andrena morio* Brullé 1832.

Ophrys iricolor has a disjunctive distribution in the Mediterranean region. In the west it can be found on Corsica, Sardinia, and in Tunisia and in the east in Greece, the Aegean islands, Turkey, Syria, Israel, and Cyprus. However, it is absent from Italy and Sicily. It is unclear whether this disjunctive distribution stems from a former wider distribution of *O. iricolor* or from convergent evolution. The striking similarity of the floral morphology in the eastern and western populations, especially the purple underside of the flower, makes the first explanation more plausible. It is also unclear why *O. iricolor* has not been able to establish any populations in Italy.

Some authors consider the Sardinian form of *O. iricolor* as a distinct species (*O. eleonorae* Devillers-Terschuren & Devillers; Devillers and Devillers-Terschuren, 1994), while others consider it as a geographical subspecies (*O. iricolor* subsp. *maxima* Terracciano; Paulus and Gack, 1999). In this publication, we will use the name *O. iricolor*.

Species determination and sample collection—Plants of *O. iricolor* and *O. lupercalis* were determined in the field according to morphological flower characters. Typical flowers of *O. iricolor* have sharp edges at the base of the labellum, a bright blue speculum, and a fully purple underside, often with a broad greenish-yellow margin. Flowers of *O. lupercalis* have a clear curve at the base of the labellum without any edges and a grayish-blue speculum. The underside has no purple. Flowers with intermediate characters, e.g., edges at the base of the labellum, but not colored on the underside or without edges but purple on the underside, were determined as hybrids.

Samples from *O. lupercalis*, *O. iricolor*, and putative hybrid plants were collected from several populations on Sardinia (Table 1). For collection of flower odor, flower labella were extracted in 1.5 ml pentane (Uvasolv, Sigma-Aldrich, Munich, Germany) for 48 h. Afterward, the labella were removed and the samples stored at −20°C. For morphometric measurements, whole flowers, including sepals and petals, were put in 70% ethanol. For collection of DNA samples, small pieces of leaves were put in bags containing silica gel (Merck, Darmstadt, Germany). We collected all three types of samples from all Sardinian populations. Additionally, we collected silica-dried plant material of *O. iricolor* from Greece (Crete and Kephallonia) and of *O. lupercalis* from Majorca. All samples were made on the same day the flowers were collected.

Behavioral experiments—Flowers were tested for their attractiveness to the pollinators of both species in the field. Flowers were collected with the stem and put in water to keep them fresh. Flowers treated in this way remain attractive to their pollinators for about one week. At locations with many patrolling males of *A. nigroaenea* and *A. morio*, flowers were put on bushes in the flight path of the patrolling males. Flowers that released pseudocopulatory behavior in the males were scored as attractive. Males of *A. morio* were found near Oristano, males of *A. nigroaenea* near Nuoro.

Chemical analyses—Prior to analysis, extracts were concentrated to 100 μL, and 1 μg octadecane (Sigma-Aldrich, Munich, Germany) was added as an internal standard. Samples were analyzed on a Thermo Trace gas chromatograph (Thermo Electron, Waltham, Massachusetts, USA) equipped with a DB5 capillary column (30 m, 0.25 mm i.d., J&W) and a flame ionization detector (FID). Helium was used as carrier gas (1.5 ml·min^{-1} constant flow). The sample (1 μL) was injected splitless at 50°C. After 1 min, the split valve was opened, and the oven temperature increased at 4°C·min^{-1} to 310°C.

Electrophysiology—In all pollination systems of *Ophrys* investigated so far, the electrophysiological active (EAD-active) compounds are also the behaviorally active compounds (Schiestl et al., 1999; Ayasse et al., 2003; Mant et al., 2005). The EAD-active compounds in the floral odor of *O. lupercalis* and *O. iricolor*, which can be detected by *A. nigroaenea* and *A. morio*, respectively, are known from previous analyses (Schiestl et al., 1999; Stökl et al., 2005, 2007). Additionally, we performed EAD recordings with floral extracts of *O. iricolor* and *A. nigroaenea* male antennae according to the method described in Stökl et al. (2005).

Morphometric analyses—Flowers were photographed under a binocular microscope with a digital camera. Seventeen flower characters were measured from these images using the program Analysis (Soft Imaging Systems, Münster, Germany). The following characters were measured: width and length of the labellum, length of the lateral lobe, width at the base of the median lobe, length of the median lobe, largest width of the median lobe, distance from the largest width to base of the median lobe, length of the central mark, width and length of the sepals, width and length of the petals, width and height of the gynostemium, width and height of the stigmatic cavity, and the space between the pollinia.

TABLE 1. Location, date, and number of samples collected from the investigated *Ophrys* species.

Population	Date (DD.MM.YY)	*O. lupercalis*	*O. iricolor*	Hybrids	GPS coordinates (UTM) Zone, East, North
Sardinia					
Tuttavista	31.03.04	3	—	1	32, 554300, 4469985
Grotta di Ispiniguli	31.03.04	18	6	2	32, 551390, 4463298
Monte Albo	01.04.04	2	20	20	32, 552785, 4489623
Monte Albo	11.04.05	4	2	17	32, 552785, 4489623
San Giovanni	02.04.04	1	6	3	32, 542134, 4459950
Domus Novas	04.04.04	1	1	3	32, 467523, 4355126
Domus Novas	09.04.05	5	2	3	32, 467523, 4355126
Siniscola	04.04.05	—	20	—	32, 557563, 4489623
Dorgali	05.04.05	12	—	—	32, 551456, 4463202
Carbonia	08.04.05	1	5	3	32, 457849, 4337664
Majorca	22.01.04–28.01.04	30	—	—	
Greece (Crete & Kephallonia)	30.03.03 & 27.03.05	—	10	—	

Genetic analyses—We used amplified fragment length polymorphism (AFLP) to investigate the genetic structure of the hybrid populations. DNA was extracted from silica-dried leaves with DNeasy Plant Mini Kits (Qiagen, Hilden, Germany) and the manufacturer's protocol. The AFLP protocol was adapted from Vos et al. (1995) with modifications as described in Schlüter et al. (2007). The preselective PCR was done using primers with one and two selective bases: *Eco*RI+A, *Mse*I+CT.

Selective PCR was done with primers with three and four selective bases. *Eco*RI primers were fluorescently labeled at the 5′-end with 6-FAM, HEX and NED. The following primer pairs were used: *Eco*RI-ACA and *Mse*I-CTGA, *Eco*RI-AGG and *Mse*I-CTAG, *Eco*RI-AGC and *Mse*I-CTCG.

PCR products were analyzed on an ABI 3100 capillary sequencer (Applied Biosystems, Foster City, California, USA) with internal size standard (ROX 500) and analyzed with the program Genescan (Applied Biosystems, Foster City, California, USA). AFLP bands were scored for presence or absence using the program Genographer (Benham et al., 1999).

Statistical analyses—Differences in the proportions of attractive and unattractive flowers were tested for significance using the χ^2-test and Fisher's exact test.

Two principal component analyses (PCAs) were performed on a set of 157 labella solvent extracts. We used arcsine-transformed values of the relative amounts of 30 EAD-active hydrocarbons for the first PCA and of 23 aldehydes and esters for a second PCA. A third PCA based on the relative amounts of the hydrocarbons was done with those 60 samples, which could be tested for attractiveness to both pollinator species. The resulting principal components (PCs) with an eigenvalue above one were used to test for differences in odor bouquets in discriminant function analyses (DFA). The standardized discriminant function coefficients and the factor loadings after varimax rotation were used to assess the importance of individual compounds. We considered a compound to have a high factor loading if the loading was above 0.5. Classification by the discriminant functions was done using the leave-one-out method. Morphometric data were analyzed in the same way. For all calculations, we used the program SPSS 13 (SPSS GmbH, Munich, Germany).

For statistical analysis of AFLP data (AMOVA, Φ_{PT}, principal coordinate analysis [PCoA], Nei's standard genetic distance) we used the program GenAlEX 6 (Peakall and Smouse, 2005). The PCoA was based on a pairwise genetic distance matrix calculated using the method of Huff et al. (1993). Φ_{PT} is analogous to F_{ST} when data are binary. F_{ST} values were estimated from the AFLP data using the program Hickory v1.0 (Holsinger and Lewis, 2003) under the *f* free model. Correlation between data sets were measured using the Mantel's test function in GenAlEX 6 (999 permutations). Maximum-likelihood hybrid indices were calculated with the program Hindex (Buerkle, 2005). Hybrid indices estimate the genetic contribution of hybridizing species to individuals of unknown ancestry. They vary from 0 to 1, where values of 0 and 1 represent the parental species.

RESULTS

Pollinator attractiveness—In 2005 we tested 33 flowers of *O. lupercalis*, *O. iricolor*, and their hybrids for their attractiveness to both *A. morio* and *A. nigroaenea* males directly in the field. Additionally, 29 flowers were tested with one of the two pollinator species. Of those flowers tested with both pollinators, all flowers of *O. lupercalis* were attractive to the pollinator of this species, *A. nigroaenea*, while 20% were also attractive to *A. morio*, the pollinator of *O. iricolor* (Fig. 1A). None of the flowers tested was attractive to *A. morio* alone. Of the flowers of *O. iricolor*, 56% were attractive to its pollinator *A. morio*, while 22% were attractive to both pollinators, and 22% attractive to *A. nigroaenea* alone. Hybrid plants were equally attractive to both pollinators, with 36% attractive to one or the other and 28% attractive to both. A χ^2-test showed a significant difference in the distribution of pollinator attractiveness between *O. lupercalis* and *O. iricolor* ($\chi^2 = 8.5$, df = 2, $P = 0.014$). Looking at the attractiveness separately for both pollinator species (including the plants tested with both pollinators), we obtained a similar result (Fig. 1B, C). Flowers of *O. iricolor* and hybrids were significantly more attractive to *A. morio* than were flowers of *O. lupercalis* (Fig. 1B, Fisher's exact test, *O. iricolor*–*O. lupercalis*: $p < 0.001$, Hybrids–*O. lupercalis*: $p < 0.05$). Flowers of *O. lupercalis* were significantly more attractive to *A. nigroaenea* than flowers of *O. iricolor* and hybrids (Fig. 1C, Fisher's exact test, $p < 0.05$).

Odor analyses—The GC-EAD analysis showed 45 peaks, comprising 51 compounds, in the labellum extracts of *O. iricolor* to be EAD-active in *A. nigroaenea* (Fig. 2, Table 2). Including the EAD-active compounds known from *A. morio* (Stökl et al., 2007), we analyzed 47 peaks, comprising 53 compounds in the floral extracts of *O. lupercalis*, *O. iricolor*, and hybrids (Table 2). A PCA based on hydrocarbons produced four principal components (PCs), explaining 72% of the total variance. The first PC had the highest factor scores for alkanes with a chain length of 21, 22, 26, 27, 28, and 29 as well as alkenes with a chain length of 23 and 25. The second PC consisted of alkanes with a chain length of 23–25 as well as alkenes and alkadienes with a chain length of 27 and 29. A DFA resulted in two discriminant functions (f1: $\chi^2 = 97.5$, df = 8, $p < 0.001$; f2: $\chi^2 = 3.1$, df = 3, $P = 0.379$). In the first function, PC 1 had the highest coefficient. The scatter plot of the two functions shows a limited separation of *O. lupercalis* from *O. iricolor* (A). Hybrids overlap with both, but more hybrid plants were placed with *O. iricolor*. In the classification, 81% of *O. lupercalis* were correctly classified, but only 32% of hybrids and 64% of *O. iricolor*. Overall, classification was correct at 57.3%.

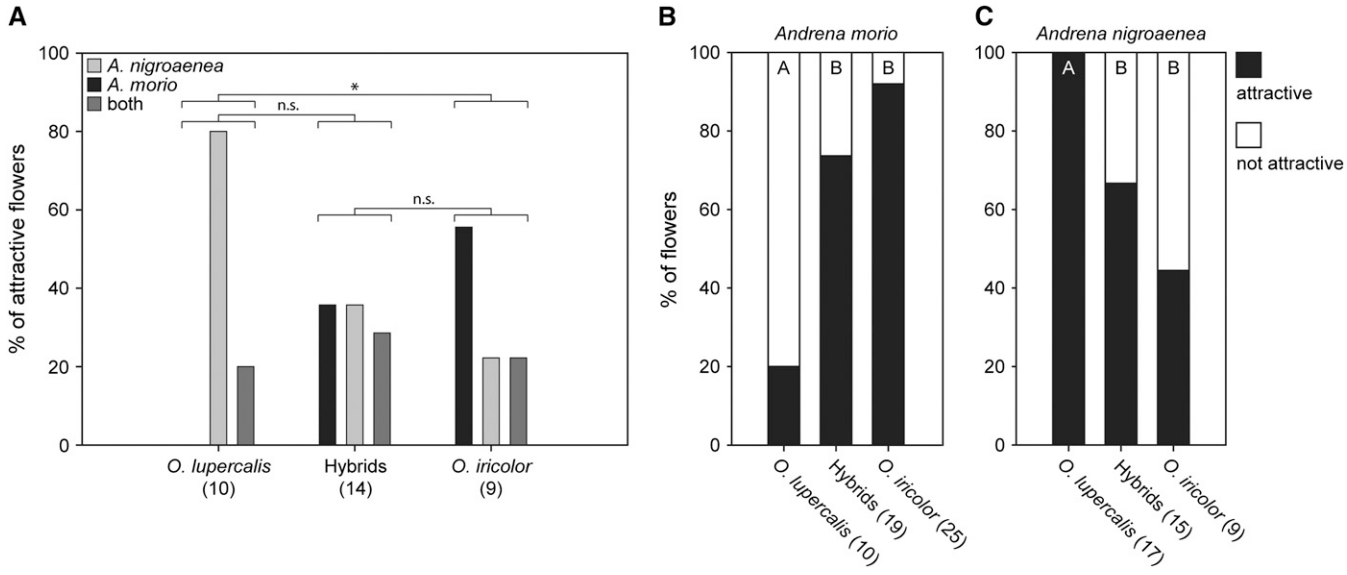

Fig. 1. (A) Attractiveness of flowers of *Ophrys lupercalis*, *O. iricolor*, and hybrids to *Andrena nigroaenea* and *A. morio*. Each flower was tested with both species. * Significant difference, χ^2-test, $p < 0.05$. Number of flowers tested in brackets. (B) Attractiveness of flowers of *O. lupercalis*, *O. iricolor*, and hybrids to *A. morio*. (C) Attractiveness of flowers of *O. lupercalis*, *O. iricolor*, and hybrids to *A. nigroaenea*. In (B) and (C), different letters indicate a significant difference between species, Fisher's exact test, $p < 0.05$. Number of flowers tested in brackets.

In a further analysis, samples were not grouped by species but by attractiveness to the pollinator species in the bioassays. The PCA produced five PCs, explaining 75% of the total variance. The same compounds as in the first analysis had the highest factor scores in the first two PCs. In the DFA, which produced two functions (f1: $\chi^2 = 40.3$, df = 8, $p < 0.001$; f2: $\chi^2 = 1.8$, df = 4, $P = 0.618$), plants that were attractive to *A. morio* were clearly separated from those attractive to *A. nigroaenea* (Fig. 3B). Samples attractive to both pollinator species were more similar to those attractive to *A. morio*. Nevertheless, two of them were also placed and classified with the samples attractive to *A. nigroaenea*. Classification was correct at 77%, with 75% of those samples attractive to both pollinators classified with those attractive to *A. morio*.

The PCA based on nonhydrocarbons produced six PCs, explaining 74% of the total variance. The first PC had the highest factor scores for aldehydes with a chain length of 9–13 and 16–20 and one unidentified compound. The second PC had the highest factor scores for aldehydes with a chain length from 22 and 24. The DFA produced two significant functions (f1: $\chi^2 = 104.9$, df = 12, $p < 0.001$; f2: $\chi^2 = 14.7$, df = 5, $p < 0.05$) and had only a weak separation of parental species (Fig. 3C). Again,

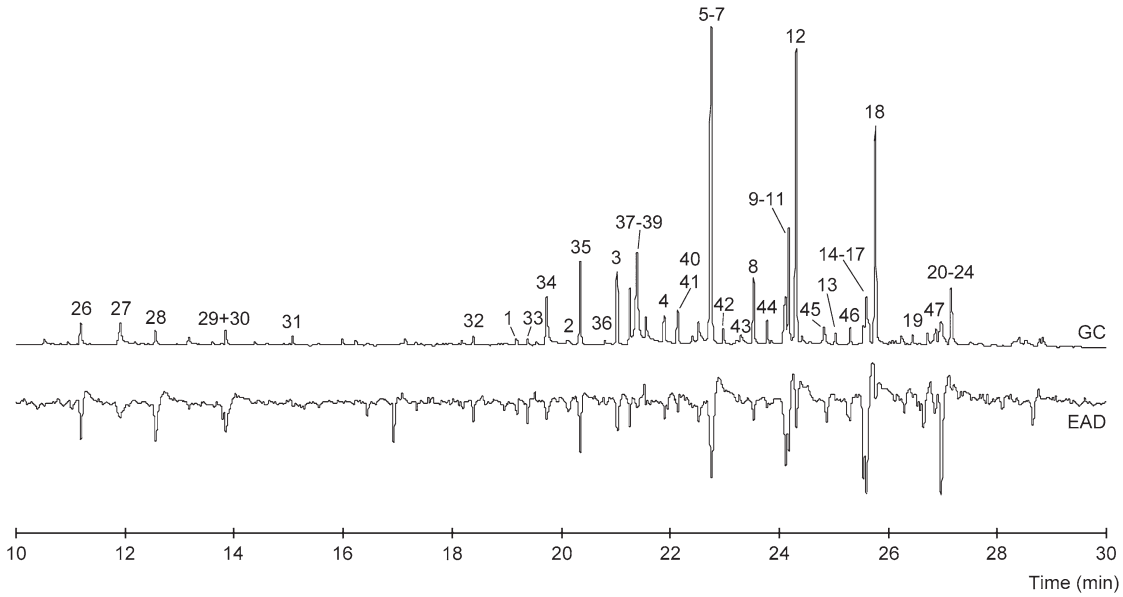

Fig. 2. Simultaneous recordings of GC and EAD signals using *Ophrys iricolor* labellum extract and *Andrena nigroaenea* male antenna. Peaks are numbered according to Table 2.

TABLE 2. Relative amounts (mean ±SE) of floral volatiles in the labella-extracts of the investigated species and EAD response of *Andrena nigroaenea* (this study) and *A. morio* (from Stökl et al., 2007). Compounds are separated into hydrocarbons and nonhydrocarbons and sorted by GC retention times. Percentage of compounds was calculated separately for hydrocarbons and nonhydrocarbons. Letters indicate significant differences (Sign.) between (A) *O. lupercalis* and hybrids, (B) *O. lupercalis* and *O. iricolor*, (C) hybrids and *O. iricolor*. n.s., no significant difference. Mann–Whitney *U* test, Bonferroni correction $p < 0.05$.

		EAD Reaction in		Percentage (mean ± SE)			
No.	Compound	*A. nigroaenea*	*A. morio*	*O. lupercalis*	Hybrids	*O. iricolor*	Sign.
Hydrocarbons							
1	Nonadecane	x	—	0.2 ± 0.12	0.2 ± 0.12	0.2 ± 0.12	n.s.
2	Eicosane	x	x	0.1 ± 0.19	0.1 ± 0.15	0.1 ± 0.12	n.s.
3	Heneicosane	x	x	1.1 ± 0.93	3.3 ± 2.32	3.3 ± 1.55	A,B
4	Docosane	x	x	0.4 ± 0.36	1.1 ± 0.77	1.0 ± 0.57	A,B
5	(*Z*)-9-Tricosene	x	x	0.2 ± 0.28	0.8 ± 0.77	1.0 ± 1.00	A,B
6	(*Z*)-7-Tricosene	x	x	0.1 ± 0.24	0.3 ± 0.33	0.4 ± 0.32	A,B
7	Tricosane	x	x	26.5 ± 9.84	32.4 ± 9.22	31.4 ± 7.28	A,B
8	Tetracosane	x	x	2.2 ± 0.90	3.2 ± 1.37	3.0 ± 1.00	A,B
9	(*Z*)-11/(*Z*)-10-Pentacosene [a]	x	x	0.9 ± 0.77	1.9 ± 1.41	3.1 ± 2.01	A,B,C
10	(*Z*)-9-Pentacosene	x	x	0.6 ± 0.58	1.3 ± 1.01	1.7 ± 1.34	A,B
11	(*Z*)-7-Pentacosene	x	x	1.0 ± 2.72	5.1 ± 3.88	6.1 ± 3.98	A,B
12	Pentacosane	x	x	11.8 ± 3.62	12.7 ± 3.68	11.9 ± 3.46	n.s.
13	Hexacosane	x	—	0.6 ± 0.23	0.5 ± 0.19	0.4 ± 0.12	A,B
14	Heptacosadiene [b]	x	—	0.1 ± 0.13	0.1 ± 0.09	0.1 ± 0.11	n.s.
15	(*Z*)-13/(*Z*)-12/(*Z*)-11-Heptacosene [a]	x	x	4.4 ± 1.91	3.1 ± 2.38	3.5 ± 2.22	A,B
16	(*Z*)-9-Heptacosene	x	x	5.8 ± 3.04	4.9 ± 3.94	4.5 ± 2.61	n.s.
17	(*Z*)-7-Heptacosene	x	x	2.4 ± 1.66	2.1 ± 1.92	2.0 ± 1.68	n.s.
18	Heptacosane	x	x	13.2 ± 3.78	9.3 ± 3.95	8.3 ± 3.11	A,B
19	Octacosane	x	—	0.6 ± 0.45	0.4 ± 0.22	0.4 ± 0.21	A,B
20	Nonacosadiene [b]	x	—	2.7 ± 1.43	1.6 ± 1.45	1.8 ± 1.33	A,B
21	Nonacosadiene [b]	x	x	6.4 ± 4.02	3.5 ± 2.46	3.6 ± 2.55	A,B
22	(*Z*)-14/(*Z*)-13/(*Z*)-12/(*Z*)-11-Nonacosene [a]	x	x	6.4 ± 4.28	4.3 ± 4.22	4.4 ± 3.62	A,B
23	(*Z*)-9-Nonacosene	x	x	5.2 ± 5.11	3.0 ± 3.43	3.2 ± 2.94	n.s.
24	Nonacosane	x	—	7.2 ± 2.46	4.7 ± 2.21	4.4 ± 1.92	A,B
Nonhydrocarbons							
25	Nonanal	x	x	7.0 ± 2.74	7.4 ± 3.02	5.1 ± 2.72	B,C
26	Decanal	x	x	2.2 ± 1.22	2.4 ± 1.29	1.6 ± 1.10	B,C
27	Unknown	x	x	3.5 ± 1.73	3.7 ± 2.23	2.6 ± 1.93	B,C
28	Undecanal	x	x	1.5 ± 0.95	1.2 ± 0.69	0.9 ± 0.59	B
29	Unknown	x	x	0.1 ± 0.10	0.1 ± 0.14	0.1 ± 0.08	n.s.
30	Dodecanal	x	x	1.8 ± 1.18	1.6 ± 0.93	1.3 ± 0.83	n.s.
31	Tridecanal	—	x	1.0 ± 0.73	10 ± 0.62	0.9 ± 0.55	n.s.
32	Hexadecanal	x	x	1.0 ± 0.72	1.2 ± 1.10	0.9 ± 0.65	n.s.
33	Heptadecanal	x	—	0.8 ± 0.50	0.9 ± 0.52	0.8 ± 0.65	n.s.
34	Unknown	x	—	12.2 ± 6.48	7.5 ± 4.43	9.1 ± 4.10	A,B
35	Octadecanal	x	x	3.6 ± 3.26	9.1 ± 6.51	6.9 ± 5.60	A,B
36	Unknown	x	x	2.4 ± 3.78	0.5 ± 0.88	0.7 ± 0.78	A,B
37	Nonadecanal	x	x	5.4 ± 3.01	7.1 ± 4.75	5.2 ± 4.52	C
38	Unknown	x	x	26.0 ± 13.15	13.4 ± 11.2	22.0 ± 16.31	A,C
39	Unknown	x	x	7.2 ± 3.48	6.9 ± 5.49	6.1 ± 3.79	n.s.
40	Unknown	x	x	0.1 ± 0.22	0.0 ± 0.05	0.0 ± 0.11	A,B
41	Eicosanal	x	x	3.8 ± 1.65	6.6 ± 3.33	5.0 ± 3.06	A,C
42	Heneicosanal	x	x	2.8 ± 1.65	2.1 ± 1.55	2.1 ± 1.71	B
43	Unknown	x	x	0.8 ± 1.36	2.7 ± 2.59	3.9 ± 3.80	A,B
44	Docosanal	x	x	4.7 ± 1.99	5.2 ± 2.26	4.2 ± 1.85	n.s.
45	2-Nonyl hexadecanoate [c]	—	x	3.0 ± 4.14	10.9 ± 8.54	13.3 ± 10.30	A,B
46	Tetracosanal	x	x	5.4 ± 3.47	5.6 ± 2.82	5.0 ± 3.27	n.s.
47	Unknown	x	—	3.8 ± 3.47	2.9 ± 2.56	2.5 ± 2.45	n.s.

[a] Compounds could not be separated with the GC parameters used.
[b] Double-bond positions unknown.
[c] Enantiomeric composition unknown.

hybrid samples were more similar to *O. iricolor* than to *O. lupercalis*, but overlapped with both of them. Classification was correct at 60.5%, with 81.0% correct for *O. lupercalis*, 55.4% for hybrids, and 50.8% for *O. iricolor*.

Morphometric analyses—We measured morphometric flower characters from 117 samples. A principal component analysis (not shown) based on all 17 characters produced four components with an eigenvalue above one, explaining 74% of the variance. Principal component one had high factor loadings for mainly those characters describing the length and width of the labellum and the median and lateral lobes. PC two had high loadings for the width of the stigmatic cavity, the width of the gynostemium, and the space between the pollinia. PC three described the width and length of the sepals and petals. A discriminant function analysis (Fig. 3D) based on the PCs produced

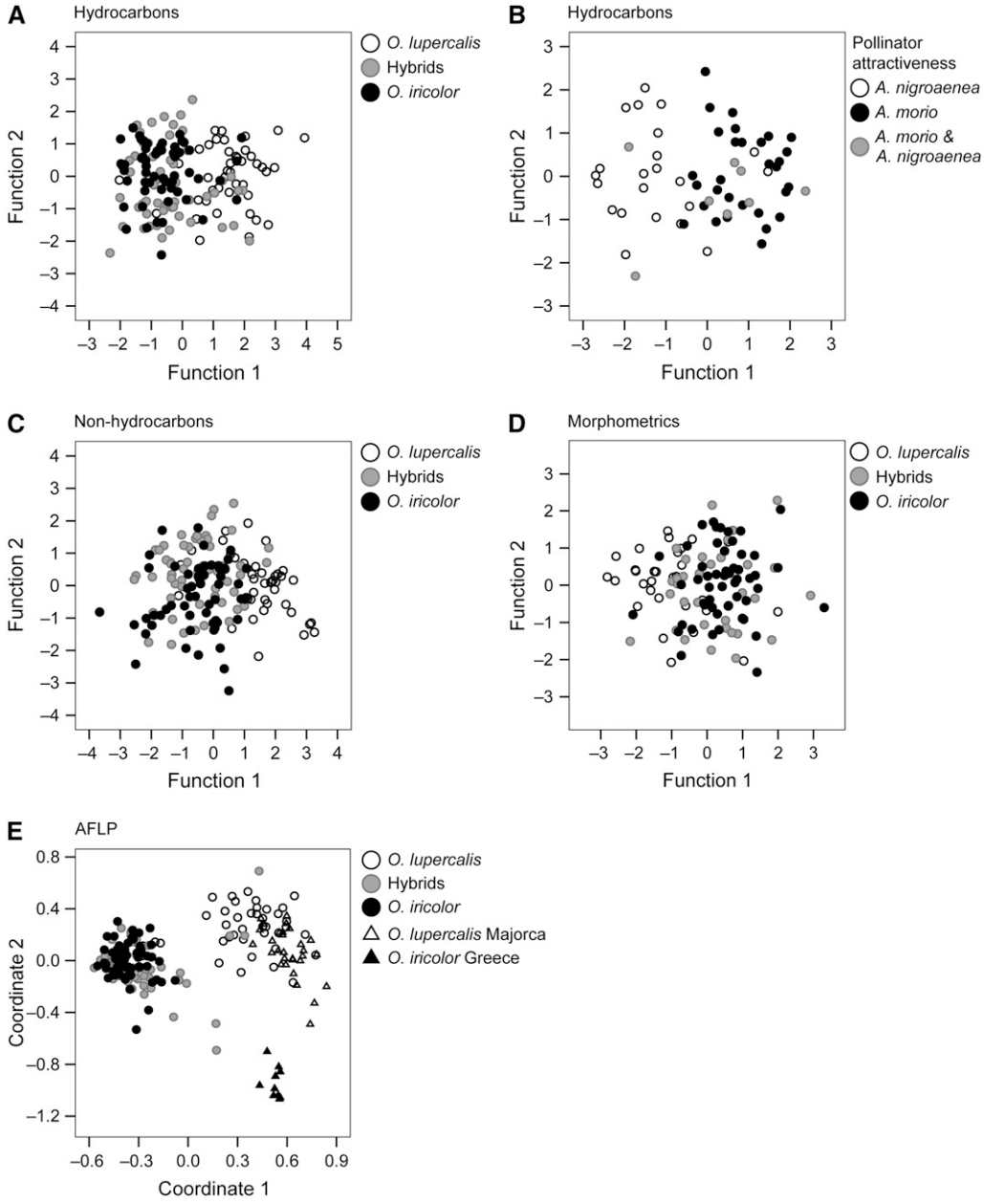

Fig. 3. Scatter plots of discriminant function analyses of (A) EAD-active hydrocarbons with samples grouped by *Ophrys* species; (B) EAD-active hydrocarbons with samples grouped by their attractiveness to *Andrena nigroaenea* and *A. morio*; (C) EAD-active nonhydrocarbons; (D) Morphological flower characters; and (E) Scatter plot of the first two coordinates from a principal coordinate analysis based on ALFP data.

two functions (f1: $\chi^2 = 36.1$, df = 8, $p < 0.001$; f2: $\chi^2 = 0.8$, df = 3, $P = 0.855$). Separation of the parental species was incomplete with a large overlap. Hybrid plants did not form a separate cluster, but were placed near the samples of *O. iricolor*. In a classification, 70% of them are placed with *O. iricolor* and 30% with *O. lupercalis*. *Ophrys lupercalis* and *O. iricolor* were correctly classified at 67.7% and 86.5%, respectively. Overall, the sample classification was correct at 56.4%.

AFLP analyses—We scored 145 AFLP bands from the three primer combinations. The mean percentage of polymorphic loci was 79.03%. The number of private bands was very low, with two private bands in *O. lupercalis* and one each in *O. iricolor*, hybrids, and *O. lupercalis* from Majorca. Values of Φ_{PT} (and estimated F_{ST}) were 0.155 (0.132) between *O. lupercalis* and *O. iricolor,* 0.128 (0.105) between *O. lupercalis* and hybrids, and 0.007 (0.017) between *O. iricolor* and hybrids. A principal coordinate analysis produced six coordinates, of which the first three explained 72.2% of variance (c1: 41.9%, c2: 19.6%, c3: 10.7%). *Ophrys iricolor* and hybrids formed a common cluster separated from *O. lupercalis* (Fig. 3E), although some samples of *O. lupercalis* were placed with *O. iricolor* and hybrids and some hybrid samples with *O. lupercalis*. Samples of *O. lupercalis* from Majorca were placed with *O. lupercalis* from Sardinia. *Ophrys iricolor* from Greece formed a separate cluster.

To measure the correlation between our data sets, we performed a Mantel test, but only for samples from Sardinia where all types of data were available (108 samples). It showed no correlation between genetic distance and flower odor (hydrocarbons: $r = 0.221$, $P = 0.001$; nonhydrocarbons: $r = 0.136$, $P = 0.006$), flower odor and morphometric data (hydrocarbons: $r = 0.09$, $P = 0.039$; nonhydrocarbons: $r = 0.035$, $P = 0.212$), and genetic distances and morphometric data ($r = 0.091$, $P = 0.042$). The test between genetic distance and geographic distance showed no correlation for *O. lupercalis* and hybrids (lupercalis: $r = 0.181$, $P = 0.076$; hybrids: $r = -0.059$, $P = 0.353$) but a slight correlation for *O. iricolor* ($r = 0.316$, $P = 0.008$).

Hybrid indices clearly showed the introgression between *O. lupercalis* and *O. iricolor* (Fig. 4A). Many intermediate genotypes can be found. The transition is almost complete with only a small gap between *O. iricolor* and hybrids on the one hand and *O. lupercalis* on the other hand. Again, *O. iricolor* and hybrids showed a huge overlap, with some putative hybrid plants genetically indistinguishable from *O. iricolor*, while some *O. iricolor* plants clearly showed introgression. The analysis including samples from Majorca and Greece showed an overlap between *O. lupercalis* from Sardinia and Majorca, while *O. iricolor* from Greece is clearly separated from the Sardinian samples (Fig. 4B).

DISCUSSION

Pollinator attractiveness and cross-pollination—Most of the flowers tested were attractive to the expected pollinator of the investigated species. Nevertheless, we also found flowers that were attractive to pollinators other than the expected pollinator, as well as plants that attracted two pollinator species. These results raise doubt about the hypothesis of complete reproductive isolation of *Ophrys* species by selective attraction of a single pollinator species, as proposed by Paulus and Gack (1990a). Nonlegitimate pollination in *Ophrys* can result in fertile hybrids (Ehrendorfer, 1980), and if backcrosses occur, gene flow across species boundaries has to be expected.

Attraction of the nonlegitimate pollinator should be more likely if the involved pollinator species are closely related and possess a similar female sex pheromone. *Andrena nigroaenea* and *A. morio* are two closely related species, both members of the subgenus *Melandrena* (Warncke, 1968). They are attracted to *Ophrys* flowers by different mixtures of the same hydrocarbons (Schiestl et al., 1999; Stökl et al., 2007). The same hydrocarbons, but again in a different bouquet, are also used by *O. bilunulata* to attract males of *A. flavipes* (Schiestl and Ayasse, 2002). In such cases, changes in the amounts of already existing compounds are sufficient to attract a different pollinator, but no new compounds have to be produced by the flowers. Therefore, *Andrena*-pollinated *Ophrys* species, as the species of the *O. fusca* group, should tend to a high rate of nonlegitimate pollination and consequently to hybridization and introgression. The high similarity of pollinator-attracting scent could have been a decisive factor for the strong radiation of this group.

Importance of various floral signals—We found 51 compounds in the floral odor of *O. iricolor* that released reactions in the antennae of *A. nigroaenea*. This number is a few more than in previous analyses (Stökl et al., 2005).

For *A. nigroaenea*, the mixture of hydrocarbons, namely alkanes and alkenes, releases pseudocopulatory behavior in the males (Schiestl et al., 1999; Schiestl and Ayasse, 2002), resulting in a high selective pressure on the bouquet of hydrocarbons. Nonhydrocarbons, however, showed a flower-specific variation of scent (Ayasse et al., 2000). Plants can thereby reduce the habituation of insect males and increase the proportion of males that visit more than one flower of an inflorescence. Therefore, the bouquet of nonhydrocarbons is not specific to the pollinator species (Stökl et al., 2005), and a greater difference than in the

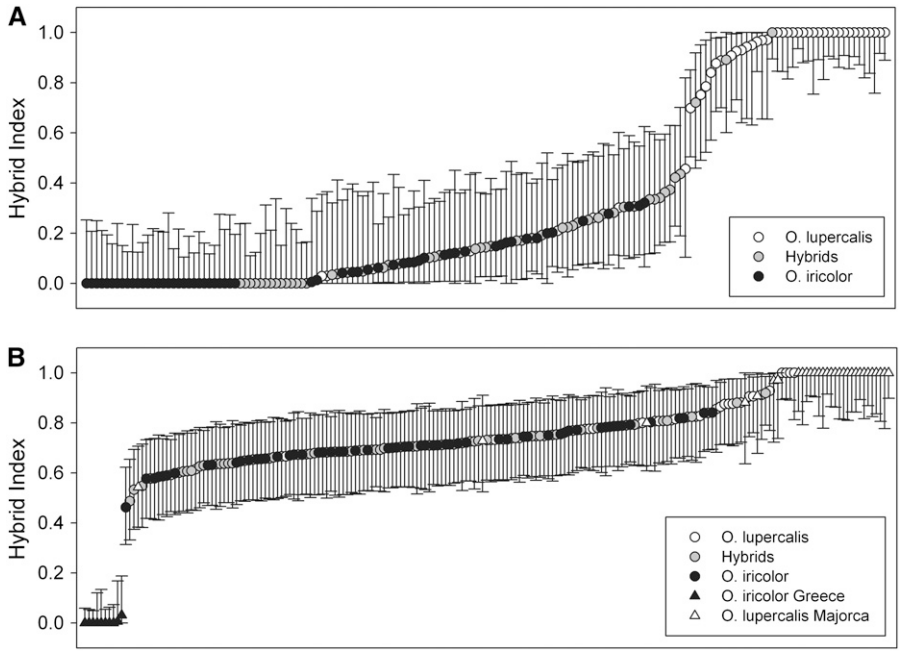

Fig. 4. (A) Hybrid indices varying from 0 = *Ophrys iricolor* to 1 = *O. lupercalis* for all Sardinian samples. (B) Hybrid indices varying from 0 = *O. iricolor* from Greece to 1 = *O. lupercalis* from Majorca for all investigated species. Whiskers give 95% confidence interval.

bouquet of hydrocarbons has to be expected. Interestingly, in our analysis the DFAs for hydrocarbons and nonhydrocarbons resulted in a similar proportion of correctly classified samples (57.3% and 60.5%, respectively). In previous studies (Kullenberg, 1961; Schiestl et al., 1999), at least in *Andrena*-pollinated systems, olfactory signals were much more important for pollinator attraction than were visual signals. However, flower morphology is important for positioning the pollinator on the flower and therefore for the correct placing of the pollinia. In our analysis, morphological flower traits showed a significant difference between the two investigated species, but with a broad overlap. The correct classification of 56.4% is comparable to the results of the analyses on floral odor.

In the analysis of Gölz and Reinhard (1990), the Sardinian population of *O. iricolor* had morphological flower characters intermediate between *O. iricolor* from Crete and *O. lupercalis* from Majorca, which were well separated. However, Gölz and Reinhard (1990) did not compare *O. lupercalis* and *O. iricolor* from Sardinia. The low difference between the two species on Sardinia, as well as the low difference in flower odor, is most likely caused by the frequent hybridization and introgression between them.

The discriminant function analysis with samples grouped by their attractiveness for the pollinators showed a good separation of flowers attractive to *A. nigroaenea* and *A. morio*. Nevertheless, there is an overlap between the two odor bouquets, and the two samples attractive to *A. nigroaenea* were placed with those attractive to *A. morio* (Fig. 2B). Flowers that were attractive to both pollinators did not form a distinct group, but had a complete overlap with the other samples. However, they seemed to be more similar to those attractive to *A. morio* as 75% were placed with them.

Genetic structure of populations—The AFLP analysis showed a good separation of the two parental species, whereas hybrids could not be separated from *O. iricolor*. Nevertheless, some individuals from both parental species were clustered with the other species. Similar results were obtained from the analysis of flower odor and morphology, although a Mantel's test showed no correlation between the data sets. We conclude from our data that care must be taken when using flower morphology to assign a plant to a taxon. Even a test for pollinator attractiveness does not always prove the identification of a plant as a given species.

Previous genetic studies on Italian and French *Ophrys* population also found low differentiation and gene flow between sympatric species (Soliva and Widmer, 2003). Mant et al. (2005) found a clear separation of floral odor bouquets in Italian species of *O. sphegodes, O. exaltata* (subsp. *archipelagi*), and *O. garganica*, whereas the species could not be separated in a microsatellite analysis. In our study, however, the analyses of floral odor and AFLPs gave very similar results. This discrepancy could be due to the higher number of markers used in our AFLP analysis and to the much higher variability of microsatellite markers used in the aforementioned study. Genetically, the Sardinian populations of *O. iricolor* are clearly distinct from populations in Greece, whereas this is not the case for populations of *O. lupercalis* from Sardinia and Majorca. Furthermore, our data showed that most putative hybrid plants are more similar to *O. iricolor* than to *O. lupercalis* (Figs. 3, 4). We therefore think that *O. iricolor* has almost been replaced by an *O. iricolor* ×*lupercalis* hybrid population on Sardinia. According to AFLP data, "pure" *O. lupercalis* can still be found, but it is not clear whether a stable genetic equilibrium has yet been reached. Competition for pollinators and the higher number of hybrid individuals make pollination events between pure individuals of *O. lupercalis* unlikely and rare. Eventually, *O. lupercalis* might be completely displaced by the *O. iricolor* ×*lupercalis* hybrid population on Sardinia. An alternative hypothesis for the distinction of the Sardinian *O. iricolor* from the Greek populations could be the lack of gene flow due to geographic isolation, rather than to hybridization. In this case, the populations of *O. iricolor* in Tunisia and Malta should be closely related to the Sardinian populations and separated from the ones from Greece. A third explanation could be convergent evolution of *O. iricolor*-type morphology in the western and eastern parts of the Mediterranean. Although convergent acquisition of the same pollinator species by different *Ophrys* species could be demonstrated in a previous study (Stökl et al., 2005), none of the alternative explanations are supported by our results, which clearly show nonlegitimate pollination and hybridization between *O. iricolor* and *O. lupercalis*. A wider genetic analysis of eastern and western *O. iricolor* populations would help explain the current pattern of distribution.

Breakdown of reproductive isolation—A large overlap of the flowering times on Sardinia could have been responsible for the high degree of hybridization. In the eastern part of the Mediterranean, *A. morio* and *A. nigroaenea*-pollinated species occur in sympatry without hybridization. On Crete, *O. iricolor* flowers from the middle of March to the middle of April. *Ophrys sitiaca*, which is pollinated by *A. nigroaenea*, flowers from February to the middle of March. So there is no overlap in the flowering period of the *A. morio*- and *A. nigroaenea*-pollinated *Ophrys* species. A different situation can be found on Sardinia. There *O. iricolor* blooms from the end of February until the middle of May and *O. lupercalis* from March to the middle of May, which results in an almost complete overlap of the flowering periods. Naturally, the activity periods of the males of the pollinator species must be the same as or at least overlap with the flowering period of the *Ophrys* species.

Smith and Ayasse (1987) and recently Vereecken et al. (2007) showed that the female sex pheromone of a bee species differs between populations and that males prefer allopatric odor types. If a new species with a slightly different flower odor is introduced into the distribution area of a species, e.g., due to a shift of the flowering period, the pollinators could be attracted by the different odor type of this second species. However, no hybrids between the two bee species have been found so far. We therefore postulate additional isolation mechanisms for the bees, such as genetic incompatibility, a different mating behavior, or morphological barriers. In the genus *Andrena*, the form of the male genitalia is a distinctive feature for many species (Schmid-Egger and Scheuchl, 1997). It is possible that this also prevents successful mating between species.

Conclusion—Our data showed the breakdown of the reproductive barriers between *O. lupercalis* and *O. iricolor* on Sardinia. This breakdown leads to introgression between the species and presumably to the displacement of both species by a hybrid population. Shifted flowering periods because of climatic changes may be the main reason for the breakdown on Sardinia and could explain the occurrence of hybrids in other *Ophrys* populations, too. Furthermore, not all males react in the same way to an odor bouquet. Factors other than floral odor that influence the male bee's behavior should be better investigated.

We also showed that nonlegitimate pollination and consequently hybridization seem to be more frequent in *Ophrys* than previously thought and that pollinator-driven selection is an important factor in the radiation of this genus.

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