Pollinator driven radiation in sexually deceptive orchids of the genus *Ophrys*

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Pseudokopulation eines *Andrena flavipes* Männchens auf

einer Blüte von *Ophrys bilunulata*.

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Table of contents

Summary

Pollinator driven radiation in sexually deceptive orchids of the genus *Ophrys*

Introduction

Terrestrial orchids of the genus *Ophrys* are pollinated by one of the most remarkable pollination mechanisms: sexual deception of male insects. *Ophrys* flowers imitate the virgin females of its insect pollinators to attracted males to the flowers. The mislead males try to copulate with the putative female and thereby they transfer pollen and pollinate the flowers (Kullenberg 1961). *Ophrys* flowers resemble the females only vaguely in shape and color, but perfectly in terms of the floral odor (reviewed in Ayasse 2006; Paulus 2006), which is identical to the female sex-pheromone of the virgin females of the pollinator species (Schiestl *et al*. 1999).

This pollination mechanism is exclusive to Orchidaceae and is known from Europe, Africa, South America, and Australia (Dafni 1984; Ackermann 1986; Nilsson 1992). In Europe only the genus *Ophrys* in pollinated by sexual deception. Pollinators of *Ophrys* flowers are mostly bees and wasps, and two beetles (Kullenberg 1961, 1973; Borg-Karlson 1990; Paulus and Gack 1990; Paulus 2006).

Decisive for a successful attraction of males to the flowers is the floral odor. In *O. speculum* attraction of males of the scoliid wasp *Campsoscolia ciliata* is achieved more or less by a single compound (Ayasse *et al*. 2003). In contrast, attract *Andrena*-pollinated *Ophrys* species their pollinators by a mixture of saturated and unsaturated hydrocarbons (Schiestl *et al*. 1999, 2000). *Ophrys lupercalis* and *O. bilunulata* attract males of their pollinators *Andrena nigroaenea* and *A. flavipes* by different bouquets of the same hydrocarbons (Schiestl and Ayasse 2002). *Ophrys* species that are pollinated by the same *Andrena* species use the same compounds in very similar composition for its attraction, independently from their phylogenetic relationship (Stökl *et al*. 2005).

In *O. sphegodes*, besides the pollinator attracting bouquet of hydrocarbons, the floral odor also contains a number of non-hydrocarbons, mostly aldehydes (Ayasse *et*

al. 2000). The bouquet of these compounds varies from flower to flower to avoid habituation (Ayasse *et al.* 2000).

Although the flower odor is the decisive factor for the attraction of the males, the flower morphology does also play an important role. After landing on a flower, morphological cues guide the males into the correct position to take off or deposit the pollinia. The current phylogeny and classification of *Ophrys* species is mainly based on morphological characters of the flowers (Delforge 2006).

Due to the highly specialized floral odor, an *Ophrys* species can normally attract only one pollinator species. The selective attraction of pollinators serves as the main reproductive isolation barrier between species (Bergström 1978; Ehrendorfer 1980; Paulus and Gack 1990). However, this mechanism does not provide a complete isolation, as hybrids between *Ophrys* species can be comparatively often found (Stebbins and Ferlan 1956; Danesch *et al*. 1975; Ehrendorfer 1980).

This type of reproductive isolation barrier may also play an important role in the evolution and radiation of *Ophrys*. If the floral scent of an odor mutant or a hybrid plant by chance resembles the female sex-pheromone of a different pollinator species, it is at the same time reproductively isolated from its parental species. Subsequently, a new species could evolve. This would be a rare example of sympatric speciation.

Thesis topic

In my thesis I investigated two putative hybrid populations of *Andrena*-pollinated *Ophrys* species: *Ophrys lupercalis*, *O. iricolor* and its hybrids on Sardinia and *O. lupercalis*, *O. bilunulata*, *O. fabrella* and hybrids on Majorca. The broader aim of the study was to learn more about the role of the floral odor in processes of speciation and radiation in *Ophrys*. In detail, I looked at the variation in floral odor and morphological flower traits and their consequences on pollinator attraction and hybridization in *Ophrys*.

The following questions were addressed:

1) Which compounds in the floral odor of an *Ophrys* species can be perceived by the males of its pollinator species?

2) Do flowers of *O. iricolor* and its model, virgin females of *A. morio*, attract males with the same compounds produced by
3) Are flowers of parental species and hybrids attractive to one or more pollinator species?
4) How much does the floral odor vary within and between species?
5) How much does the flower morphology vary within and between species?
6) What is the genetic variation within and between species?
7) Is hybridization and introgression genetically detectable?
8) Is there a correlation between phenotype (floral odor and morphology) and genotype?

Investigated species

O. iricolor DESFONTAINES shows a disjunctive distribution in the Mediterranean. It can be found in Corsica, Sardinia, Tunisia, and Malta in the west as well as in Greece, the Aegean islands, Turkey, Syria, and Israel in the east (Delforge 2006). Some authors consider the eastern and western populations as separate (sub-) species (Delforge 2006), although in both areas of distribution the flowers are pollinated by *A. morio* BRULLE 1832.

O. lupercalis DEVILLERS-TERSCHUREN & DEVILLERS is widespread in the western and central part of the Mediterranean. It can be found from Portugal to Italy. It is pollinated by males of *A. nigroaenea* KIRBY 1802.

O. bilunulata RISSO shows a very similar area of distribution as *O. lupercalis*, but does not occur on Corsica and Sardinia. It has smaller flowers than *O. lupercalis* and is pollinated by *A. flavipes* PANZER 1799.

O. fabrella PAULUS & AYASSE is pollinated by *A. fabrella* PEREZ 1903 and is endemic to the Balearic Islands.

Methods used

To get a complete dataset, we combined behavioral experiments, electrophysiology, chemical and morphological analyses, and populations genetics.

In behavioral experiments I presented individual flowers to males of the pollinator species. Flowers that attracted males and released a pseudocopulatory behavior were scored as attractive to this pollinator species.

Compounds in the floral odor, that can be perceived by the pollinating males were identified with gas-chromatography coupled with an electroantennographic detector (GC-EAD). To compare odor bouquets between *Ophrys* species the relative amounts of the EAD-active compounds were used for multivariate statistics. Analysis of floral odor was separately performed for hydrocarbons and non-hydrocarbons.

Flower morphology was investigated by measuring 17 floral characters on ethanol preserved material. Obtained data were analyzed using multivariate statistics.

The genetic structure of the investigated populations was investigated using Amplified Fragment Length Polymorphism (AFLP) and appropriate statistics.

Summary Chapter 1

In this study I compared the floral scent of *O. iricolor* with the female sex-pheromone of its pollinator *A. morio* and identified the compounds that can be perceived by the pollinating males.

The floral odor of *Ophrys* flowers mimics the female sex-pheromone of its pollinator species. Schiestl *et al*. (1999) could show for the first time the identity of the sex-pheromone of virgin *A. nigroaenea* females and floral scent of *O. sphegodes* flowers. Since then the chemical signals of flowers and virgin pollinator females of two more *Ophrys* – pollinator relationships were analyzed: *O. speculum* pollinated by *Campsoscolia ciliata* (Ayasse *et al.* 2003) and *O. exaltata* pollinated by *Colletes cunicularius* (Mant *et al.* 2005a). In *O. speculum*, pollinator attraction is achieved more or less by a single, orchid-released compound, which also proved to be the major component of the sex pheromone released by females of the scoliid wasp, *C. ciliata*. In contrast, in *Andrena* pollinated *Ophrys* species the males are attracted by species-specific blends of straight chain hydrocarbons, mostly alkanes and alkenes (Schiestl *et al.* 1999; Schiestl and Ayasse 2002; Stökl *et al*. 2005).

Compounds in the flower extracts of *O. iricolor* and cuticle washes of *A. morio* females were identified with GC-MS. To identify the compounds, that can be perceived by males of *A. morio,* we performed gas-chromatography with an electroantennographic detector.

Overall, 38 peaks, comprising 41 chemical compounds were found to release reactions in the antennae of *A. morio* males (Fig. 1). Compounds were mainly straight chain alkanes and alkenes with 20 – 29 carbon atoms, aldehydes (C9 - C24), and two wax-type esters. Almost all of those compounds were found in similar amounts in both, the surface extracts of *A. morio* females and *O. iricolor* labellum extracts (Table 1). Due to the low number of samples of *A. morio*, only a rough quantification, but no statistical analysis on the relative amounts of the compounds could be done.

In the cuticle extracts of *A. morio* females and flower extracts of *O. iricolor* the same patterns of alkanes, alkenes and aldehydes were found to release responses in the antenna of *A. morio* males. Since in *Andrena* pollinated *Ophrys* species studies performed so far proved the GC-EAD active hydrocarbons present on the surface of females and flowers to be active in male attraction (Schiestl *et al*. 1999; Mant *et al*. 2005a), we expect at least the EAD-active hydrocarbons identified in *O. iricolor* and *A. morio* to be behaviorally active in the bees.

Almost all of those compounds that released signals in the antennae of *A. morio* are also EAD-active in *A.* nigroaenea and *A. flavipes* (Schiestl and Ayasse 2002; Stökl *et al*. 2005). Apart from the hydrocarbons we found a row of straight chain aldehydes as well as a mixture of two esters to be EAD-active. In *O. sphegodes* the same aldehydes and esters showed a flower-specific variation (Ayasse *et al*. 2000) and were found to have a function to minimize learned avoidance of the flowers, and increase the likelihood that a given pollinator would visit additional plants within the same population.

Summary Chapter 2

The objective of this study was to investigate the role of hybridization and introgression in processes of speciation in the sexually deceptive orchids *O. lupercalis* and *O. iricolor* on Sardinia. Hybrid plants 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, (3) behavioral experiments with single flowers to show its attractiveness for pollinators of both parent species.

Paulus and Gack (1995) found hybrids of *O. lupercalis* and *O. iricolor* on Sardinia that were attractive to both pollinator species. Typically *O. lupercalis* and *O. iricolor*, pollinated by *A. nigroaenea* and *A. morio*, respectively, can be found in some populations in Sardinia. However, in certain populations plants can be found that are thought to be hybrids and that show intermediate morphological flower characters. Flowers of plants in such populations were attractive to the pollinators of both species.

Odor changes as a result of genetic drift, negative frequency depended selection, or hybridization may be a driving forces for speciation in *Ophrys*, since the attraction of a new pollinator by a mutant or by hybrids may act as a pre-zygotic isolation barrier (Paulus and Gack 1990). *Andrena nigroaenea* and *A. morio* are attracted by different bouquets of the same hydrocarbons (Chapter 1). False pollination and hybridization is more likely in species that attract their pollinators with different mixtures of the same compounds, as it is the case in *Andrena* pollination-systems, than is systems were attraction of different pollinators is based on qualitatively different odors. A slight shift in the amounts of some compounds can lead to the attraction of a different pollinator. No new compounds have to be produced by the plant.

The populations of *O. lupercalis* and *O. iricolor* in Sardinia provide excellent conditions to test these hypotheses in the field. On Sardinia, are large number of plants with intermediate flower characters could be found. Those were preliminary classified as hybrids.

Behavioral experiments showed approx. 20% of the flowers from both species and hybrids to be attractive to the 'wrong' or both pollinator species. The analysis of the EAD-active hydrocarbons in the floral odor showed an overlap in the floral odor of *O. lupercalis* and *O. iricolor*, whereby hybrid individuals could not be separated from *O. iricolor*. The floral odor of flowers that were attractive to both pollinator species was not intermediate between species. Those plants did also not form a common group. Analysis of morphological flower characters gave a similar result as the one of floral odor. The AFLP analysis confirmed the hybridization between species. Plants of *O. iricolor* and hybrids are genetically indistinguishable and form

an *O. iricolor – lupercalis* mix population. No pure plants of *O. iricolor* can be found in Sardinia, while *O. lupercalis* can still be found in its pure form.

Our data evidently show the breakdown of the reproductive isolation barrier between *O. lupercalis* and *O. iricolor*. The reasons for this are most probably: 1) the similarity of the pollinator attracting odor, which only shows quantitative, but no qualitative, differences 2) an overlap of the flowering period of the two species in Sardinia. As a consequence, we expect pure plants of both species to be displaced by the *O. iricolor – lupercalis* hybrid population in the future due to the ongoing hybridization events.

Summary Chapter 3

The aim of this study was to compare the pollinator attracting floral signals of *O. lupercalis*, *O. bilunulata*, and *O. fabrella* with behavioral assays, electrophysiology (GC-EAD), chemical, and morphological analyses and to investigate if *O. fabrella* evolved by radiation from *O. bilunulata*.

On Majorca, three species of the *Ophrys fusca-lutea* group occur sympatrically. *Ophrys lupercalis* pollinated by *A. nigroaenea*, *O. bilunulata* pollinated by *A. flavipes*, and *O. fabrella* pollinated by *A. fabrella*. While *O. lupercalis* and *O. bilunulata* have a very similar area of distribution in the western and central part of the Mediterranean, *O. fabrella* is endemic to the Balearic Islands and is thought to have derived from either *O. lupercalis* or *O. bilunulata.* Morphological floral characters and the late flowering period indicate a closer relatedness to *O. bilunulata*, than to *O. lupercalis.* The current hypothesis is, that *O. fabrella* evolved from late flowering *O. bilunulata* plants, which recruited a new pollinator species, *A. fabrella*. On Majorca, these three species bloom consecutively: *O. lupercalis* blooms from end of December to March. *O. bilunulata* has a flowering period from February to the mid of April. *Ophrys fabrella* is flowering from the end of March to the end of April. Therefore, the flowering period of *O. bilunulata* overlaps with that of *O. lupercalis* in February and with that of *O. fabrella* from the end of March to the mid of April. As *O. lupercalis* and *O. bilunulata* use very similar odor bouquets to attract their pollinators (Schiestl and Ayasse 2002; Stökl *et al.* 2005) and flowering periods overlap, we expected hybridization between species.

On Majorca we found only very few plants that showed intermediate morphological flower characters and could not be assigned to a species. Electrophysiological and chemical analysis showed that *A. fabrella* is attracted to *O. fabrella* by the same compounds, but in a different bouquet as *A. nigroaenea* and *A. flavipes*. A multivariate analysis of the EAD-active hydrocarbons showed significant differences between the odor bouquets of each species, but with an overlap between all of them.

In the behavioral experiments, 96.5% of the flowers of *O. bilunulata* that were tested released pseudocopulatory behavior in males of *A. flavipes*. We were not able to perform tests for attractiveness with the other *Andrena* and *Ophrys* species. Also flowers of *O. bilunulata* located area, where odor bouquets of species overlap, were attractive to males of *A. flavipes*, suggesting cross-pollination between species. The bouquets of EAD-active non-hydrocarbons are also specific for each species, but do also overlap. In a discriminate function analyses *O. fabrella* was more similar to *O. bilunulata* than to *O. lupercalis*. Therefore we assume that it evolved by radiation from *O. bilunulata*. Analysis of 17 morphological flower characters of *O. lupercalis* and *O. bilunulata* showed only minor differences between species, indicating a much lower selective pressure on flower morphology than on pollinator attracting odor.

Summary Chapter 4

In this study I used Amplified Fragment Length Polymorphism (AFLP) as a neutral genetic marker to investigate the genetic population-structure of *O. lupercalis, O. bilunulata,* and *O. fabrella* on Majorca and to asses the rate of hybridization and introgression between those species. Like in the analyses of floral scent one of the aims was to study the evolution of *O. fabrella*.

Our data show a clear separation of *O. lupercalis* and *O. fabrella*. Plants of *O. bilunulata* did not form a common group in the AFLP analysis, but were placed with either *O. lupercalis* or *O. fabrella.* Calculation of hybrid indices proofed the hybridization of *O. bilunulata* with both other species. This is in strong contrast to the distinct floral odor of *O. bilunulata* found in previous studies. However, the odor bouquets of all three species do show some overlap. The analysis showed a higher intraspecific variation of the floral odor in *O. bilunulata*, than in *O. lupercalis* and

O. fabrella. Hybrid indices also showed a slight correlation with the date of sample collection. *Ophrys bilunulata* blooms between *O. lupercalis* and *O. fabrella* and its flowering period overlaps with the one of both other species.

Discussion

Pollinator attracting odor

Males of all four species of *Andrena* that do have a function as pollinators of *Ophrys* and were investigated so far (*A. nigroaenea*, *A. flavipes*, *A. morio*, *A. fabrella*) are attracted to *Ophrys* flowers by the same set of hydrocarbons (Schiestl *et al.* 1999; Schiestl and Ayasse 2002; Stökl *et al.* 2005, Chapter 1, Chapter 3). Almost all of those compounds are perceived by the antennae of all four species. Therefore, the differences between species are only in the amounts of compounds. This bouquet based specificity of pollinator attraction was and is an important precondition for processes of speciation in the *Ophrys fusca-lutea* species group. However, this is not limited to *Ophrys* species pollinated by bees of the genus *Andrena*, as *Colletes cunicularius* is also attracted by a bouquet of hydrocarbons by *O. lupercalis*.

Attractiveness to pollinators and crosspollination

On Sardinia we found flowers of *O. iricolor, O. lupercalis* and their hybrids to be attractive to males of the "wrong" both pollinator species. Additionally, some flowers were attractive to both pollinator species. This results in cross-pollination and the formation of hybrids. Unfortunately, we could not determine key compounds of the floral odor responsible for the attraction of the 'wrong' or both pollinator species. The most obvious explanation for the attraction of two pollinator species would have been in floral odor intermediate between both species. But this was not the only explanation for cross attraction and false pollination according to the results of our chemical analyses. It is also possible that males react differently to the same *Ophrys* flower. Possible factors influencing the males' behavior could be previous encounters with females or *Ophrys* flowers in connection to frequency dependant selection, other competing males at the same location, or the age of the males. Both aspects could lower the threshold of a male to react on the floral scent.

On Majorca we were not able to test *Ophrys* flowers for their attractiveness to all pollinator species. We could only test flowers of *O. bilunulata* for their attractiveness to its pollinator *A. flavipes*. In the analysis of floral odor we found an overlap in the odor bouquets of the species. As flowers of *O. bilunulata* located in the area of overlap were also attractive to *A. flavipes*, we can expect those males to be also attracted by flowers of *O. lupercalis* or *O. fabrella* that are also located in the overlap zone. The higher intraspecific variation of the flora odor in *O. bilunulata* also indicates a higher introgression of *O. bilunulata*, than of *O. lupercalis* and *O. fabrella*. This could be proved by the genetic analysis, which evidently shows hybridization between species.

Genetic population structure and hybridization

The AFLP analysis clearly showed hybridization between species on Sardinia as well as on Majorca. In both cases, not a distinct group of (F1-) hybrids intermediate between both parental species was found, but a high number of introgressive individuals. Overall, the genetic differentiation between species was very low indicated for example by the low number of AFLP bands private to a single species.

On Sardinia, we could show that the Sardinian populations of *O. iricolor* are distinct from the eastern populations in Greece. No differences between Sardinian and Majorcan populations were found for *O. lupercalis*. Therefore, no more pure plants of *O. iricolor* can be found on Sardinia. The still pure plants of *O. lupercalis* will be probably also displaced by the hybrid population in the future because of ongoing hybridization.

Our results are in accordance with other studies on the genetics of *Ophrys* populations that also revealed gene flow between species. Soliva and Widmer (2003) used microsatellites to investigate several species of the *O. sphegodes* group. They report lower genetic differences between geographically distant populations of the same species, than between sympatric populations of different species. Their data furthermore indicate gene flow across species boundaries in this group. However, the number of actual hybrids seems to be rather low for these species. Three species of the *O. sphegodes* group were also investigated for differences in floral odor and genotype (Mant *et al.* 2005b). Here, again, only very low genetic differences were found. However, species differed in the floral odor.

Hybrids are also known from other Mediterranean orchid genera, as for example *Orchis* or *Anacamptis* (Pellegrino *et al.* 2000, 2005). Hybrids between *Anacamptis morio* and *A. papilionacea* do not produce fertile seed, thus no gene flow across species boundaries occurs (Moccia *et al.* 2007).

Hybridization and flowering time

In both investigated Ophrys systems the major reason for hybridization is the overlap of flowering time of the sympatrically occurring species that is linked to the overlap of the activity periods of the pollinators. On Crete, *O. iricolor* occurs sympatrically with another *Ophrys* species pollinated by *A. nigroaenea*, *O. sitiaca*, but flowering periods do not overlap. On Sardinia, in contrast, the flowering periods of *O. iricolor* and *O. lupercalis* do overlap, which, in combination with the similar floral odor, results in cross-pollination and hybridization. Why flowering periods are shifted on Sardinia remains, however, unclear. Climatic differences between the two islands are one possibly reason.

On Majorca, hybridization is also linked with overlapping flowering periods. We found specimen of *O. bilunulata* that bloom at the same time then *O. lupercalis* to hybridize with it. Plants of *O. bilunulata* that are flowering together with *O. fabrella* later in the year hybridize with that species. *Ophrys lupercalis* and *O. fabrella* do not overlap in their flowering periods and we could not find any sign for hybridization between those two species.

Correlation of phenotype and genotype

Although we got similar results by the analyses of the floral scent and the genetic analyses of *O. iricolor* and *O. lupercalis* on Sardinia, there was no significant correlation between these two datasets. The same is true for the populations on Majorca. Here the situation is more obvious. The analysis of floral odor gave a completely different result than the analysis of the genetic population structure. While we found a distinct floral odor bouquet of *O. bilunulata*, the samples of *O. bilunulata* did not form a unique group in the genetic analysis. A similar situation was found for *O. sphegodes* and *O. exaltata* in Italy. The floral odor was highly

differentiated, but the genetic differences within and between populations were very low (Mant *et al.* 2005b).

A highly selective pressure on the plants to maintain the correct odor for pollinator attraction despite hybridization is the only explanation for these results.

Conclusion

I my thesis I could show, that the pollinator attracting odor is one of the crucial factors influencing hybridization between *Ophrys* species. I found high rates of hybridization and introgression between species, so we can assume that this does play an important role for processes of radiation in *Ophrys*, especially for *Andrena*-pollinated species. To clarify why some plants are attractive to two pollinators, more bioassays are necessary.

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Zusammenfassung

Sympatrische Artbildung durch Bestäuber-basierte Selektion bei Sexualtäuschorchideen der Gattung *Ophrys*

Sexualtäuschorchideen der Gattung *Ophrys* imitieren mit ihren Blüten die Weibchen ihrer Bestäuber, meist Hymenopteren, um Männchen zur Bestäubung anzulocken. Die angelockten Männchen versuchen sich in einer so genannten Pseudokopulation mit den Blüten zu paaren, wobei diese bestäubt werden. *Ophrys*-Blüten imitieren die Weibchen ihrer Bestäuber in Form, Farbe und im Duft. Entscheidend für eine erfolgreiche Anlockung der Bestäuber ist der Duft der Blüte, welcher, soweit bislang gezeigt wurde, identisch ist mit dem weiblichen Sexualpheromon des Bestäubers. Durch die Artspezifität der Sexualpheromone der Bestäuber kann im Regelfall jede *Ophrys*-Art nur eine Bestäuber-Art anlocken, worauf auch die reproduktive Isolation von *Ophrys* beruht.

Im Fall der Anlockung von *Andrena nigroaenea* durch *O. sphegodes* besteht dieser Duft aus einer komplexen Mischung von ungesättigten und gesättigten Kohlenwasserstoffen (Alkane und Alkene). Verschiedene, von *Andrena* bestäubte *Ophrys*-Arten wie z.B. *O. bilunulata* oder *O. lupercalis* locken die Männchen mit denselben Substanzen, aber unterschiedlichen Mischungsverhältnissen, an. Diese Bouquet-basierte spezifische Anlockung einer einzigen Bestäuberart spielt eine wichtige Rolle bei der Artbildung von *Ophrys.* Wird der Blütenduft durch Mutation oder Hybridisierung verändert, kann ein neuer Bestäuber rekrutiert werden. Da ein neuer Bestäuber als Isolationsfaktor agiert, kann es in weiterer Folge zur Entstehung einer neuen Art kommen.

In meiner Arbeit untersuchte ich Mechanismen der sympatrischen Artbildung bei zwei von *Andrena* besuchten *Ophrys* – Bestäuber Systemen mittels Verhaltenstests, chemischen Analysen in Kombination mit Elektrophysiologie (GC-EAD), morphologischen Untersuchungen und genetischen Analysen (AFLPs).

Folgende Fragen sollten dabei beantwortet werden:

1) Wie groß ist die intra- und interspezifische Variation im Blütenduft?
2) Welche Substanzen werden zur Anlockung der verschiedenen Bestäuberarten verwendet?
3) Wie variieren morphologische Blütenmerkmale?
4) Wie hoch ist die genetische Variabilität?
5) Kommt es zur Hybridisierung zwischen den Arten?
6) Sind Hybriden und Elternarten für beide beteiligte Bestäuberarten attraktiv?

Auf Sardinien untersuchten wir Hybridpopulationen zwischen *O. lupercalis* und *O. iricolor* (siehe Manuskripte 1 und 2). Im Verhaltensexperiment waren Blüten beider Elternarten zu 20% bis 40% für den Bestäuber der jeweils anderen Art attraktiv. Hybridpflanzen waren zu gleichen Teilen für beide Bestäuber attraktiv. Bei beiden Elternarten, als auch bei Hybridpflanzen, wurden Pflanzen gefunden, die für beide Bestäuber gleichzeitig attraktiv waren.

Die elektrophysiologischen Untersuchungen zeigten, dass Männchen von *A. morio* dieselben Substanzen wahrnehmen können, wie Männchen von *A. nigroaenea*. Die Blütendüfte von *O. iricolor* und *O. lupercalis* unterscheiden sich nur quantitativ. Eine Diskriminanzanalyse, durchgeführt mit den relativen Anteilen der GC-EAD aktiven Verbindungen des Blütendufts, zeigte eine deutliche Trennung der beiden Elternarten, *O. lupercalis* und *O. iricolor*, wobei die Duftbouquets jedoch deutlich überlappen. Der Duft der Hybridpflanzen überlappte mit beiden Elterarten, war jedoch dem von *O. iricolor* ähnlicher. Entgegen unserer Erwartung, war der Blütenduft von Pflanzen, die im Verhaltenstest für beide Bestäuber attraktiv waren, nicht intermediär zwischen den beiden Elternarten. Diese Pflanzen bildeten in der Analyse auch keine separate Gruppe, sondern waren mit beiden Elternarten vermischt.

Die Analyse der Morphologie der Blüten zeigte sehr ähnliche Ergebnisse, wie die Analyse des Blütendufts.

Bei der genetischen Analyse mittels AFLP-Markern konnte ich eindeutig Introgression zwischen den beiden Arten nachweisen. Die meisten Hybrid Pflanzen bildeten eine gemeinsame Gruppe mit *O. iricolor*, während *O. lupercalis* davon abtrennt war. Allerdings kam es auch hier zur Überlappung der beiden Gruppen.

Ein Vergleich der Sardinienpopulationen mit Pflanzen von *O. iricolor* und *O. lupercalis*, die außerhalb von Sardinien gesammelt wurden, zeigte, dass es keine genetisch „reinen" Pflanzen von *O. iricolor* auf Sardinien gibt, sondern nur noch eine *O. iricolor-lupercalis* Hybridpopulation. Typische, genetisch nicht von anderen Populationen differenzierte, Pflanzen von *O. lupercalis* kommen auf Sardinien noch vor. Die größere Zahl der Hybridpflanzen und die Konkurrenz um Bestäuber führen zu einer geringeren Bestäubungsrate zwischen reinen Pflanzen von *O. lupercalis*, weshalb wir vermuten, dass *O. lupercalis* in ihrer reinen Form auf Sardinien durch die Hybridpopulation von *O. lupercalis* und *O. iricolor* verdrängt werden wird.

Die Gründe für die Hybridisierung sind die sehr ähnlichen Blütendüfte und eine Überlappung der Blühperioden von *O. lupercalis* und *O. iricolor,* bedingt durch die Aktivität der Bestäuberarten. Auf Kreta kommt *O. iricolor* ebenfalls sympatrisch mit der von *A. nigroaenea* bestäubten *O. sitiaca* vor. Da aber auf Kreta die Blühperioden der beiden Arten nicht überlappen, kommt es dort auch nicht zur Hybridisierung.

Auf Mallorca kommen drei von *Andrena* bestäubte *Ophrys* Arten vor. *Ophrys lupercalis* wird von *A. nigroaenea* bestäubt, und blüht als Erste der drei Arten, *O. bilunulata* wird von *A. flavipes* bestäubt und hat eine überlappende Blühphase mit *O. lupercalis* und *O. fabrella,* welche von *A. fabrella* bestäubt wird und zuletzt blüht. Wir untersuchten die Populationen der drei Arten mit denselben Methoden wie die Populationen auf Sardinien (siehe Manuskripte 3 und 4).

Die GC-EAD Analysen zeigten, dass auch *A. fabrella* Männchen von denselben Kohlenwasserstoffen, aber in unterschiedlichen Mischungsverhältnissen, angelockt werden, die auch die Männchen von *A. nigroaenea* und *A. flavipes* anlocken. Ein Vergleich des Blütendufts mittels einer Diskriminanzanalyse ergab signifikant unterschiedliche Duftbouquet, die sich jedoch auch überlappten.

Verhaltensexperimente konnten nur mit *O. bilunulata* und Männchen von *A. flavipes* durchgeführt werden und ergaben, dass auch Pflanzen von *O. bilunulata* mit leicht verschiedenen Duftbouquets für *A. flavipes* attraktiv waren. Auch Pflanzen aus dem Überlappungsbereich mit *O. lupercalis* und *O. fabrella* lockten die Männchen an. Daraus kann indirekt geschlossen werden, dass auch Pflanzen von *O. lupercalis* und *O. fabrella* aus dem Überlappungsbereich für *A. flavipes* attraktiv wären. Dies hätte Falschbestäubungen und Hybridisierung zur Folge.

Die postulierte Hybridisierung von *O. bilunulata* sowohl mit *O. lupercalis* als auch *O. fabrella* konnte bei den genetischen Analysen eindeutig nachgewiesen werden. Während *Ophrys lupercalis* und *O. fabrella* gut voneinander isoliert sind, wurden die Pflanzen von *O. bilunulata* zum einen Teil mit *O. lupercalis* und zu einem weiteren Teil mit *O. fabrella* gruppiert. Dieses Ergebnis steht im starken Kontrast zu der Analyse des Blütendufts, die eigenständige Duftbouquets für alle drei Arten zeigte.

Meine Ergebnisse demonstrieren, dass die reproduktive Isolation bei sympatrisch vorkommenden *Ophrys* Arten nicht so perfekt ist, wie bisher angenommen wurde. In beiden untersuchten *Ophrys*-Bestäuber Systemen konnten wir Hybridisierung und Introgression zwischen den Arten feststellen. Die Gründe dafür sind die geringen, meist nur quantitativen, Unterschiede im Duftbouquet der Arten. Leichte Veränderungen in den produzierten Mengen der einzelnen Substanzen reichen aus, um einen anderen Bestäuber anzulocken. Der zweite, ebenso wichtige Grund ist die Überlappung der Blühperioden, die erst einen Transfer von Pollen zwischen den Arten erlaubt.

Unsere Untersuchungen zeigen, dass *Ophrys* Modellorganismen für Untersuchungen zu sympatrischen Artbildungsprozessen basierend auf Bestäuberselektion darstellen.

1

Comparison of the flower scent of the sexually deceptive orchid *Ophrys iricolor* and the female sex pheromone of its pollinator *Andrena morio*

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Keywords—*Ophrys iricolor, Andrena morio*, Pollination, Sexual deception, Sex pheromone, GC-EAD.

Abstract—*Ophrys* flowers mimic the female produced sex pheromone of their pollinator species to attract males for pollination. The males try to copulate with the putative female and thereby pollinate the flower. Using electrophysiological and chemical analyses, floral volatiles released by *O. iricolor* as well as the female sex pheromone of its pollinator species, *Andrena morio* are investigated.

Overall, 38 peaks comprising 41 chemical compounds, were found to release reactions in the antennae of male *A. morio* bees. Analyses using coupled gas chromatography-mass spectrometry revealed the presence of alkanes and alkenes with 20 to 29 carbon atoms, aldehydes (C9 to C24) and two esters. Almost all of those compounds were found in similar proportions in both, the floral extracts of *O. iricolor* and cuticle surface extracts of *A. morio* females. The pattern of biologically active volatiles described here is very similar to that used by other *Ophrys* species pollinated by *Andrena* males.

Introduction

Sexually deceptive orchids of the genus *Ophrys* are pollinated by male Hymenoptera, which are not rewarded by the flower with pollen or nectar. Instead, the flower mimics the sex pheromone of the pollinators' female. Once landed on the flower the males try to copulate with the putative female, whereby the flowers are pollinated.

Sexual deception in orchids was first described by Pouyanne in 1917. Kullenberg (1961) recognized the importance of the olfactory signals for pollinator attraction and release of pseudo-copulation behavior. Schiestl *et al.* (1999) could show for the first time the identity of the sex-pheromone of virgin *Andrena nigroaenea* females and floral scent of *O. sphegodes* flowers. Since then the chemical signals of flowers and virgin pollinator females of two more *Ophrys* – pollinator relationships were analyzed: *O. speculum* pollinated by *Campsoscolia ciliata* (Ayasse *et al.* 2003) and *O. exaltata* pollinated by *Colletes cunicularius* (Mant *et al.* 2005). In *O. speculum*, pollinator attraction is achieved more or less by a single, orchid-released compound, 9-hydroxydecanoic acid, which also proved to be the major component of the sex pheromone released by females of the scoliid wasp, *C. ciliata*. In contrast, in *Andrena* pollinated *Ophrys* species the males are attracted by species-specific blends of straight chain hydrocarbons, mostly alkanes and alkenes (Schiestl and Ayasse 2002; Stökl *et al.* 2005). *Ophrys* species that are pollinated by the same bee species independent of the phylogenetic relationship were found to use the same odor bouquet for attraction (Stökl *et al.* 2005).

Although the pollinators of many *Ophrys* species are known, only the three systems mentioned above have been investigated by comparing the composition of floral volatiles with the sex-pheromone of virgin females. In this study we identified the electrophysiologically active compounds in the floral scent of *O. iricolor* and showed a striking congruence with the pattern of volatiles released by the female of its pollinator, *A. morio*.

Material and Methods

Investigated species

Ophrys iricolor DESFONTAINES belongs to the *O. fusca-lutea* group and shows a disjunctive distribution in the Mediterranean. It can be found in Corsica, Sardinia, Tunisia, and Malta in the west as well as in Greece, the Aegean islands, Turkey, Syria, and Israel in the east (Delforge 2006). Some authors consider the eastern and western populations as separate (sub-)species (Delforge 2006), although in both areas of distribution the flowers are pollinated by *A. morio*.

Andrena morio BRULLÉ 1832 is a relatively large (females 17-18 mm, males 13-14 mm), species of the subgenus *Melandrena* with totally black colored individuals. Females nest solitary in sandy soil. The species is bivoltine, with a first generation in spring and a second generation in late summer (Schmid-Egger and Scheuchel 1997).

Sample collection

Samples from *O. iricolor* (n=59) and females of *A. morio* (n=2) were collected in Sardinia in March 2005. Flowers were collected with the stem and put in a vial with water to keep them fresh. Flowers treated that way remain attractive to pollinators for several days. At places with many patrolling males of *A. morio* the flowers were put in the flight patch of a male. Flowers that attracted and released the pseudocopulatory behavior in a male were scored as attractive. Females of *A. morio* were put in a cage (approx. 40x40x40 cm) containing 5 to 10 males of *A. morio*. Females that released copulatory behavior in the males were scored as attractive. Both, flowers of *O. iricolor* and females of *A. morio*, were positively tested for their attractiveness to males of *A. morio*. For collection of flower volatiles, labella were extracted in 1.5 ml pentane (Sigma-Aldrich, Uvasol) for 48 hours. Females of *A. morio* were freeze killed and subsequently extracted for 30 sec in 1.5 ml pentane. All samples were concentrated and stored at -20°C until analysis.

Electrophysiology

To identify those compounds in the complex flower sent that are perceived by the male bees' antenna we used gas chromatography coupled with an electroantennographic detector (GC-EAD) according to the described method (Stökl *et al.* 2005). GC-EAD active compounds were investigated by coupled gas chromatography/mass spectrometry (GC/MS).

Structure elucidation

All samples were analyzed using a gas chromatograph series 8000 linked to a Fisons MD 800 mass spectrometer (Fisons Instruments, Ismaning, Germany); 70 eV mass spectra were taken in EI mode. Helium served as carrier gas. Separations were performed using a 30 m CP8912 VF-1 MS (Varian, Darmstadt, Germany) fused silica column (i.d. 0.25 mm; film th. 0.25 μm). The temperature was initially kept at 60°C for 5 min. and then increased by 10°C per min to 300°C. Identification of compounds was carried out by comparison of mass spectra and retention times of natural products with corresponding data of synthetic reference samples. Double bond positions in alkenes were determined by investigation of the corresponding dimethyl disulfide adducts (DMDS) (Buser *et al.* 1983). Structures suggested by literature data (McLafferty and Stauffer 1989) were verified by independent syntheses: aldehydes were obtained by oxidation of the corresponding alcohols, while esters were prepared from the acid chlorides and appropriate alcohols according to laboratory standard. Syntheses of alkenes were carried out via the corresponding alkynes (Brandsma 1988) and Lindlar-hydrogenation. Structure assignments were based on coinjection of natural extracts and synthetic reference compounds.

Results

Overall, 38 peaks, comprising 41 chemical compounds were found to release reactions in the antennae of A. morio males (Fig. 1). Compounds were mainly straight chain alkanes and alkenes with 20 – 29 carbon atoms, unbranched aldehydes (C9 - C24), and two wax-type esters. Almost all of those compounds were found in similar amounts in both, the surface extracts of A. morio females and O. iricolor labellum extracts (Table 1). Since the heptacosenes showing double bonds in positions 11, 12, and 13 could not be separated, we don't know whether all three compounds are perceived by the insects' antennae, however, structure determinations are unambiguous because the DMDS-adducts (Buser et al. 1983) were found to be present. Similarly, biological activity could not be distinctly assigned to one or both of the inseparable esters, while the structures of the two compounds could be unambiguously determined on the basis of their mass spectra - even in the

mixture (Francke *et al.* 2000). The amounts of 2-nonyl hexadecanoate proved to be too small to quantify single enantiomers by enantioselective gas chromatography. Due to the low number of samples of *A. morio*, no statistical analysis on the relative amounts of the compounds could be applied. Nevertheless, a rough quantification of the components is provided.

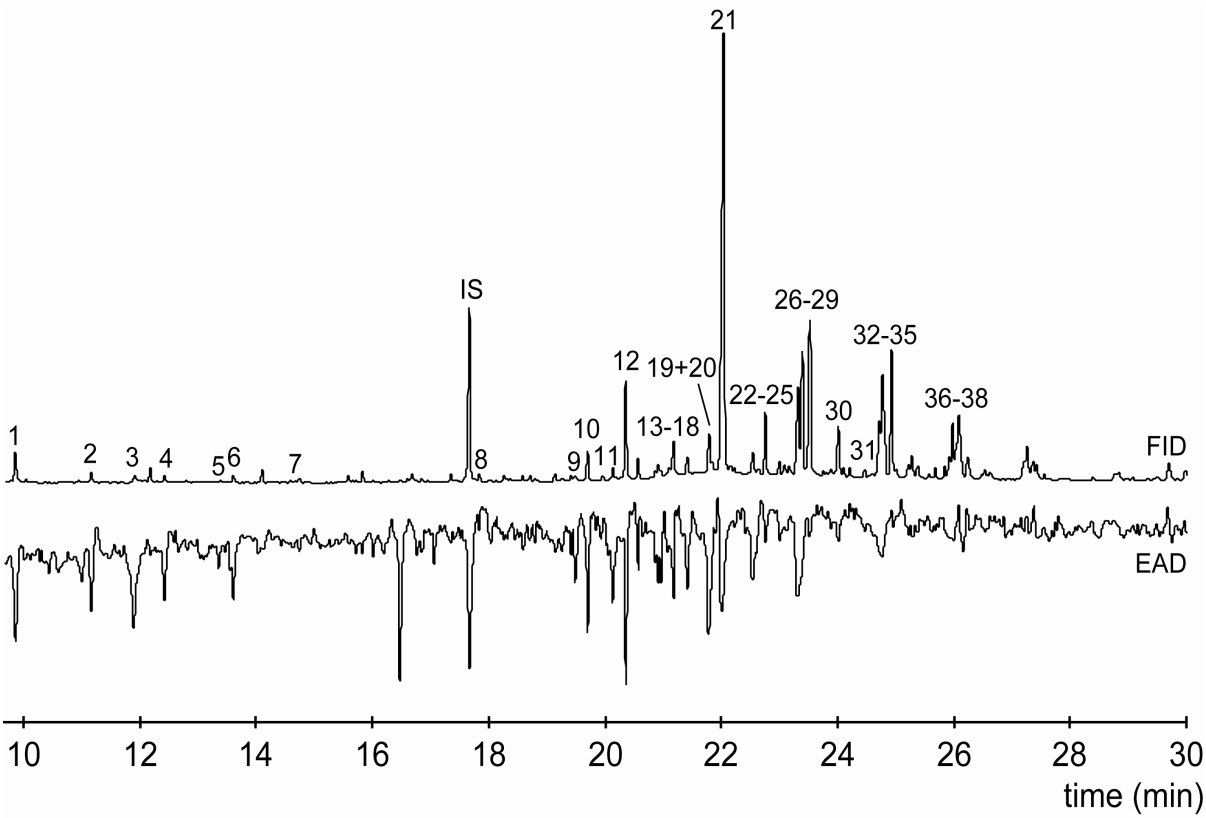

Figure 1—Conventional GC and coupled GC-EAD to detect active compounds in flower extracts of *O. iricolor* using an antenna of an *A. morio* male. Peaks are numbered corresponding to Table 1. IS – internal standard

Discussion

In the cuticle extracts of *A. morio* females and flower extracts of *O. iricolor* the same patterns of alkanes, alkenes and aldehydes were found to release responses in the antenna of *A. morio* males. Since in *Andrena* pollinated *Ophrys* species studies performed so far proved the GC-EAD active hydrocarbons present on the surface of females and flowers to be active in male attraction (Schiestl *et al.* 1999; Mant *et al.* 2005), we expect at least the EAD-active hydrocarbons identified in *O. iricolor* and *A. morio* to be behaviorally active in the bees.

Table 1—Relative amounts (A>20%, B<20%, C<5%, D<1%, -: not detected) of the compounds in the cuticle surface extracts of *A. morio* females and *O. iricolor* flowers that were active in GC-EAD analyses using *A. morio* male antennae. Compounds are sort according to their elution on a non-polar column.

* Compounds could not be separated with the GC-parameters used

** Double-Bond positions unknown

No.	Compound	*A. morio*	*O. iricolor*
1	Nonanal	-	D
2	Decanal	-	D
3	unknown	-	D
4	Undecanal	-	D
5	unknown	-	D
6	Dodecanal	-	D
7	Tridecanal	-	D
8	Hexadecanal	D	D
9	Eicosane	D	D
10	Octadecanal	D	C
11	unknown	-	D
12	Heneicosane	D	C
13	Nonadecanal	D	C
14	unknown	-	D
15	unknown	-	D
16	Docosane	D	D
17	unknown	-	D
18	Eicosanal	D	C
19	(*Z*)-9-Tricosene	D	D
20	(*Z*)-7-Tricosene	D	-
21	Tricosane	A	A
22	Heneicosanal	D	D
23	unknown	-	D
24	n-Tetracosane	C	C
25	Docosanal	D	D
26	(*Z*)-11-Pentacosene	C	D
27	(*Z*)-9-Pentacosene	D	D
28	(*Z*)-7-Pentacosene	D	C
29	Pentacosane	A	B
30	2-Nonyl hexadecanoate/Octyl hexadecanoate*	-	C
31	Tetracosanal	D	D
32	13-,12-,11-Heptacosene*	C	C
33	(*Z*)-9-Heptacosene	B	B
34	(*Z*)-7-Heptacosene	C	C
35	Heptacosane	A	B
36	Nonacosadiene**	D	C
37	(*Z*)-11-Nonacosene	B	C**
38	(*Z*)-9-Nonacosene	C	C**

Almost all of those compounds that released signals in the antennae of *A. morio* are also EAD-active in *A. nigroaenea* and *A. flavipes* (Schiestl *et al.* 1999; Stökl *et al.* 2005). Compared to the *Ophrys* species pollinated by *A. nigroaenea* and *A. flavipes* (Stökl *et al.* 2005) we found higher amounts of tricosane and tricosene and lower amounts of heptacosene and nonacosene in *O. iricolor*.

Apart from the hydrocarbons we found a row of straight chain aldehydes as well as a mixture of 2-nonyl hexadecanoate and octyl hexadecanoate to be

electrophysiologically active. In *O. sphegodes* the same aldehydes and esters showed a flower-specific variation (Ayasse *et al.* 2000) and were found to have a function to minimize learned avoidance of the flowers, and increase the likelihood that a given pollinator would visit additional plants within the same population.

In Sardinia, *O. lupercalis*, pollinated by *A. nigroaenea* and *O. iricolor*, occur in sympatric populations. If the specificity of the attractive signal is exclusively based on quantitative differences of volatiles, cross-attraction of pollinators and speciation may occur. Because of the similarities in the bouquets of both species, slight changes in relative amounts of some compounds may lead to attraction of a 'false' but successful pollinator with the consequence of hybridization. Indeed, hybrid swarms have been described, and we are presently studying the evolution of both *Andrena* pollinated *Ophrys* systems in Sardinia.

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2

Scent variation and hybridization cause the displacement of a sexually deceptive orchid species on Sardinia

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Keywords—AFLP, *Andrena*, GC-EAD, chemical analysis, hybridization, *Ophrys*, pollination, sexual deception

Abstract—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. To assess the role of odor changes in the radiation and speciation of this genus we investigated hybrid swarms of *O. lupercalis* and *O. iricolor* on Sardinia using behavioral, electrophysiological (GC-EAD), chemical, morphological, and genetic methods (AFLPs).

Behavioral experiments showed that approx. 20% of the flowers from both species and hybrids were attractive to the 'wrong' or both pollinator species. The analysis of the EAD-active hydrocarbons in the floral odor showed an overlap in the odor bouquets of the two species, whereby hybrid individuals could not be separated from *O. iricolor*. A very similar result was obtained by the analysis of 17 morphological flower characters. The AFLP analysis confirmed the hybridization

between species. Plants of *O. iricolor* and hybrids are genetically indistinguishable and form an *O. iricolor – lupercalis* hybrid population. No pure plants of *O. iricolor* can be found in Sardinia. *O. lupercalis* can still be found in its pure form on Sardinia, but it will possibly be displaced by the *O. iricolor – lupercalis* hybrid population in the future.

Introduction

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; Ackermann 1986; Nilson 1992). The sexually deceptive genus *Ophrys* is spread throughout the whole Mediterranean, but can also be found in central and northern Europe, and consists of more than 200 species (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, 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, both closely and distantly related *Ophrys* species that are pollinated by the same *Andrena* species use identical compounds in a very similar composition for pollinator attraction (Stökl *et al.* 2005). This indicates a convergent evolution of pollinator attracting odors in *Ophrys* species.

Normally, an *Ophrys* species attracts only one pollinator species. The high degree of specialization in pollination serves as main reproductive isolation between sympatric *Ophrys* species, which are mostly 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, false pollination does occur and hybrids can be found frequently (Stebbins and Ferlan 1956; 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 frequency dependent selection, or hybridization may be the driving forces for speciation, since the attraction of a new pollinator by a mutant or by the hybrids may act as a pre-zygotic isolation barrier (Paulus and Gack 1990a).

False 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. Due to selection by the new pollinator, such plants may adapt to a new ecological niche and a new species may evolve.

The pollinator-mediated isolation between *Ophrys* species might be not as complete as previously expected. 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 pollinator species. Typical *O. lupercalis* and *O. iricolor*, pollinated by *A. nigroaenea* and *A. morio*, respectively, can be found in some populations on Sardinia. However, in certain

populations plants that are thought to be hybrids and that show intermediate characters can be found. 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 a successful pollination. Morphological investigations of the Sardinian populations of *O. lupercalis* and *O. iricolor*, as well as *O. lupercalis* from Majorca and *O. iricolor* from Crete, showed the Sardinian population to be 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 to genetic structure of (hybrid) populations and make it possible to assess the gene flow between species.

The objective of this study was to investigate the role of hybridization and introgression in processes of speciation in the sexually deceptive orchids *O. lupercalis* and *O. iricolor* on Sardinia. 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, (3) behavioral experiments with single flowers to test the attractiveness to pollinators of both parent species.

Materials and Methods

Study species

O. 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 the section Pseudophrys. Several species within the *fusca-lutea* group are morphologically very similar and therefore difficult to classify (Paulus 2001; Delforge 2006). *O. 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.

O. iricolor has a disjunctive distribution in the Mediterranean. In the west it can be found on Corsica, Sardinia, and in Tunisia; in the eastern part in Greece, the Aegean islands, Turkey, Syria, Israel, and Cyprus. However, it is absent from Italy and Sicily. It is unclear whether these separate areas of distribution were once connected or evolved independent through convergent evolution. The striking similarity of the flower morphology in the eastern and western population, especially the purple underside of the flower, makes the first explanation more probable. 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 other consider it 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* show sharp edges at the base of the labellum, a bright blue speculum, and a fully purple colored underside, often with a broad greenish-yellow margin. Flowers of *O. lupercalis* have a clear bend at the base of the labellum without any edges and a grayish-blue speculum. The underside shows no purple color. Flowers with intermediate characters, e.g. edges at the base of the labellum, but no colored underside or no edges and a purple color, 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 (Sigma-Aldrich, Uvasolv) for 48 hours. Afterwards 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 at the same day the flowers were collected.

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)
Sardinia:					Zone, East, North
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	-	

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) was added as internal standard. Samples were analyzed on a Thermo Trace gas chromatograph (Thermo Electron, Waltham, USA) equipped with a DB5 capillary column (30m, 0.25mm i.d., J&W) and a FID. Helium was used as carrier gas (1.5 ml min^{-1} constant flow). 1µl of the sample was injected splitless at 50°C. After one minute the split valve was opened and the oven temperature increased at 4°C min^{-1} to 310°C.

Electrophysiology

In all *Ophrys* pollination systems investigated so far, the electrophysiological active (EAD-active) compounds also were the behavioral 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. Sixteen flower characters were measured from these pictures using the 'Analysis' software (Soft Imaging Systems, Münster, Germany). The following characters were taken: Length of the labellum, length of the lateral lobe, width of the labellum, 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 of the sepals, length of the sepals, width of the petals, length of the petals, width of the gynostemium, width of the stigmatic cavity, space between the pollinia, height of the gynostemium.

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 3 and 4 selective bases. *Eco*RI-Primers were fluorescently labelled at the 5'-end with 6-FAM, HEX and NED. 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, USA) with internal size standard (ROX 500) and analyzed with Genescan Software. AFLP bands were scored for presence or absence using the Genographer software (Benham 1999).

Statistical analysis

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 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 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 an analogous to F_{ST} when data are binary. F_{ST} values were estimated from the AFLP data using Hickory v1.0 (Holsinger and Lewis 2003) under the f free model. UPGMA trees were constructed with Splitstree 4 (Huson 1998). Correlation between data sets were measured using the Mantel's test function of GenAlEX 6 (999 permutations). Maximum-likelihood hybrid indices were calculated with 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. Additional 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. 56% of the flowers of *O. iricolor* 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 each of them 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* (χ^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 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 52 compounds, in the labellum extracts of *O. iricolor* to be EAD-active in *A. nigroaenea* (Fig. 2, Table 2). Together with the EAD-active compounds known from *A. morio* (Stökl *et al.* 2007), we analyzed 47 peaks, comprising 54 compounds in the flower extracts of *O. lupercalis*, *O. iricolor*, and hybrids (Table 2). A PCA based on hydrocarbons produced 4 principal components (PC), 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 to 25 as well as alkenes and alkadienes with a chain length of 27 and 29. A DFA resulted in 2 discriminant functions (f1: χ^2=97.5, df=8, p<0.001; f2: χ^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* (Fig. 3A). 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%.

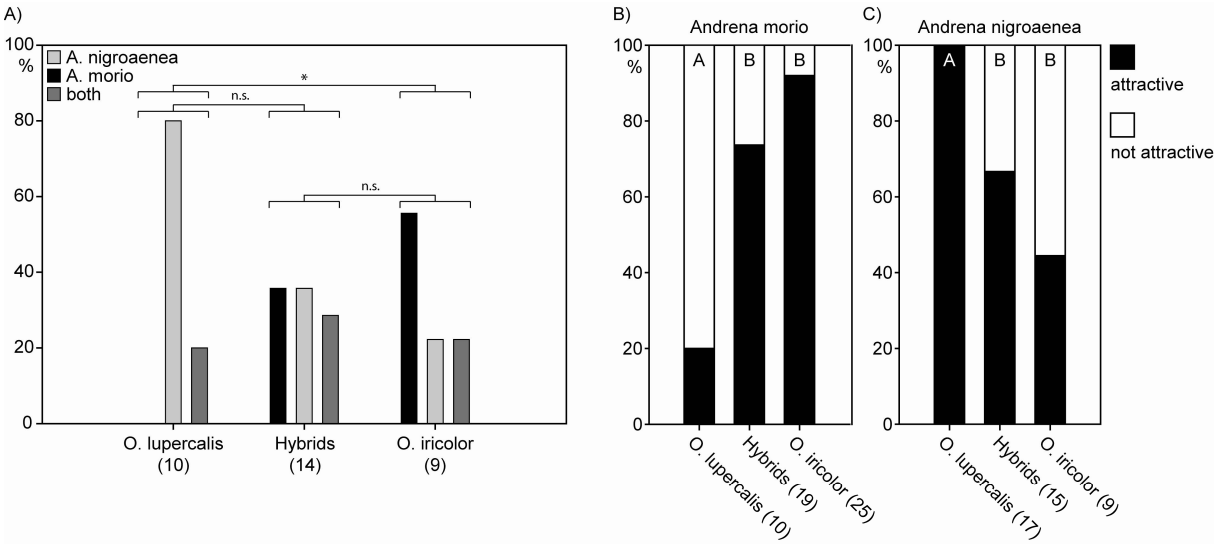

Figure 1—A) Attractiveness of flowers of *O. lupercalis*, *O. iricolor*, and hybrids to *A. nigroaenea* and *A. morio*. Each flower was tested with both species. * significant difference, χ^2-test, p<0.05. Number of flowers tested in brackets. B) Attractiveness of flowers of *O. lupercalis*, *O. iricolor*, and hybrids to *A. morio*. C) Attractiveness of flowers of *O. lupercalis*, *O. iricolor*, and hybrids to *A. nigroaenea*. 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 5 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: χ^2=40.3, df=8, p<0.001; f2: χ^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 non-hydrocarbons produced 6 PCs, explaining 74% of the total variance. The first PC had the highest factor scores for aldehydes with a chain length of 9 to 13 and 16 to 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: χ^2=104.9, df=12, p<0.001; f2: χ^2=14.7, df=5, p<0.05) and showed only a weak separation of parental species (Fig. 3C). Again, 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*.

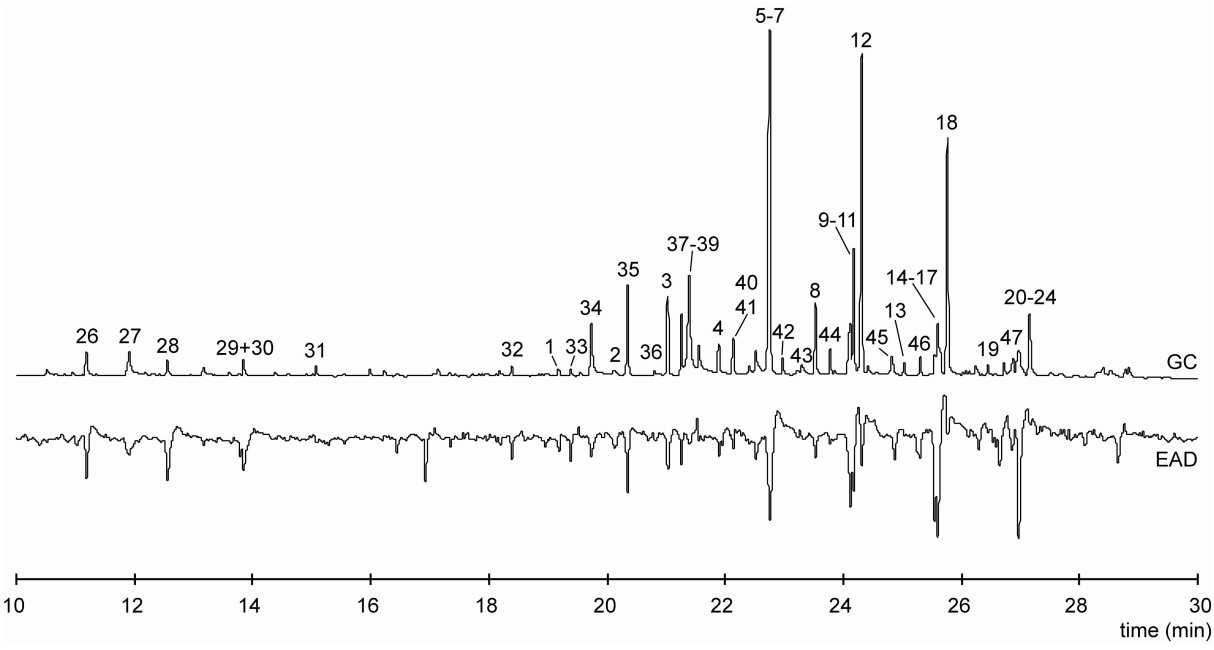

Figure 2—Simultaneous recordings of GC and EAD signals using *O. iricolor* labellum extract and *A. nigroaenea* male antenna. Peaks are numbered according to table 2.

Table 2—Relative amounts (Mean ±SE) of floral volatiles in the labella-extracts of the investigated species and EAD response of *A. nigroaenea* (this study) and *A. morio* (from Stökl *et al.* 2007). Compounds are separated into hydrocarbons and non-hydrocarbons and sorted by GC-retention times. Percentage of compounds was calculated separately for hydrocarbons and non-hydrocarbons. Letters indicate significant differences 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 $p \leq 0.05$. [1] Compounds could not be separated with the GC parameters used. [2] Double-bond positions unknown. [3] Enantiomeric composition unknown. x1 to x9 = unidentified compounds.

			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 [1]	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 [2]	x	-	0.1±0.13	0.1±0.09	0.1±0.11	n.s.
	15	(*Z*)-13/(*Z*)-12/(*Z*)-11-Heptacosene [1]	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 [2]	x	-	2.7±1.43	1.6±1.45	1.8±1.33	A,B
	21	Nonacosadiene [2]	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 [1]	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

Table 2—continued

Non-hydrocarbons	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	X 1	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	X 2	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	X 3	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	X 4	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	X 5	x	x	26.0±13.15	13.4±11.2	22.0±16.31	A,C
	39	X 6	x	x	7.2±3.48	6.9±5.49	6.1±3.79	n.s.
	40	X 7	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	X 8	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 [3]	-	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	X 9	x	-	3.8±3.47	2.9±2.56	2.5±2.45	n.s.

Morphometric analysis

We measured morphometric flower characters from 117 samples. A principal component analysis (not shown) based on all 17 characters produced 4 components with an eigenvalue above one, explaining 74% of the variance. Principal component one showed high factor loadings for mainly those characters describing the length and width of the labellum and the median and lateral lobes. PC two showed 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 two functions (f1: χ^2=36.1, df=8, p<0.001; f2: χ^2=0.8, df=3, p=0.855). Separation of the parental species is incomplete with a large overlap. Hybrid plants do not form a separate cluster, but are placed near the samples of *O. iricolor*. In a classification, 70% of them are placed with *O. iricolor* and 30% with *O. lupercalis*. *O. lupercalis* and *O. iricolor* were correctly classified at 67.7% and 86.5%, respectively. Overall, the sample classification was correct at 56.4%.

AFLP analysis

We scored 145 AFLP bands from 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 in each *O. iricolor*, hybrids, and *O. lupercalis* from Majorca. UPGMA tress constructed from Φ_{PT} values and Nei's genetic distances are shown in Fig. 4. The lowest values were found between *O. iricolor* and hybrids. *O. lupercalis* is equally distant from *O. iricolor* and hybrids, but closer to *O. lupercalis* from Majorca. *O. iricolor* from Greece is clearly distant from all Sardinian samples. Estimated F_{ST} values were 0.1316 between *O. lupercalis* and *O. iricolor* and 0.1051 between *O. lupercalis* and hybrids. Between *O. iricolor* and hybrids F_{ST} was 0.0168. A principal coordinate analysis produced 6 coordinates, of which the first three explained 72.2% of variance (c1: 41.9%, c2: 19.6%, c3: 10.7%). *O. iricolor* and hybrids form 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. *O. iricolor* from Greece form a separate cluster.

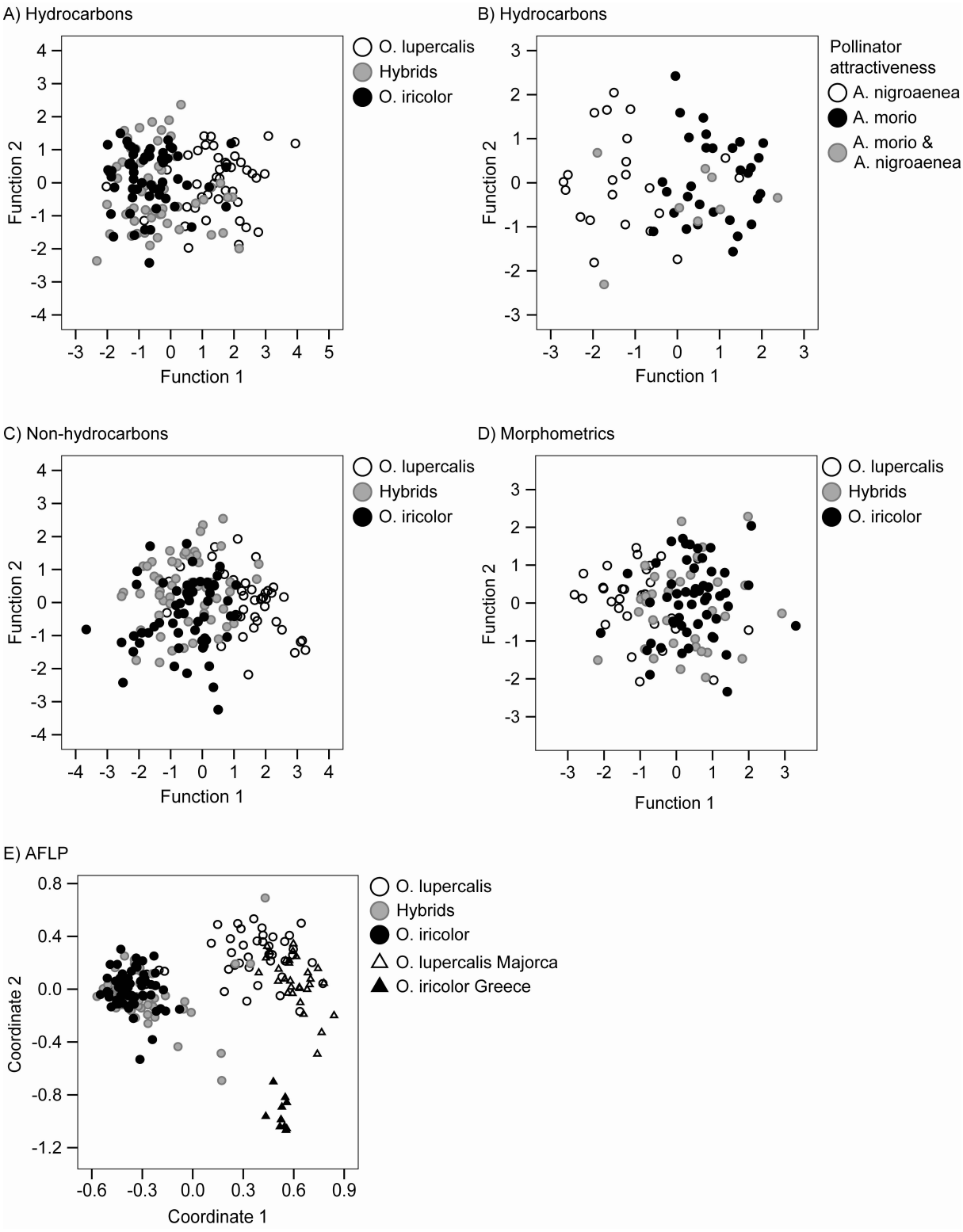

Figure 3—Scatter plots of discriminant function analyses of A) EAD-active hydrocarbons with samples grouped by *Ophrys* species B) EAD-active hydrocarbons with samples grouped by their attractiveness to *A. nigroaenea* and *A. morio* C) EAD-active non-hydrocarbons D) Morphological flower characters and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

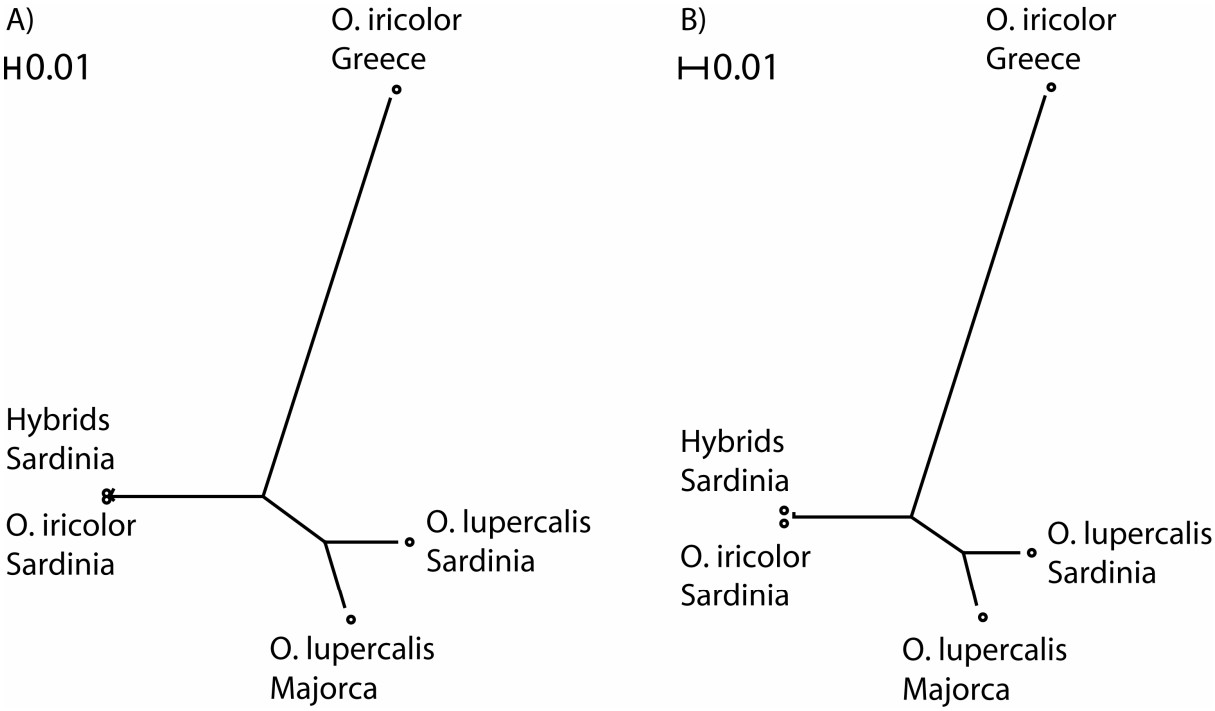

Figure 4—UPGMA trees constructed from a pairwise matrix of A) Φ_{PT} values and B) Nei's standard genetic distance.

To measure the correlation between our datasets we performed a Mantel test. This was only done for samples from Sardinia were 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; non-hydrocarbons: r=0.136, p=0.006), flower odor and morphometric data (hydrocarbons: r=0.09, p=0.039; non-hydrocarbons: 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 weak correlation for *O. iricolor* (r=0.316, p=0.008).

Hybrid indices clearly show the introgression between *O. lupercalis* and *O. iricolor* (Fig. 5A). 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 show a huge overlap, with some putative hybrid plants genetically indistinguishable from *O. iricolor*, while some *O. iricolor* plants clearly show introgression. The analysis including samples from Majorca and Greece shows an overlap between *O. lupercalis* from Sardinia and Majorca, while *O. iricolor* from Greece is clearly separated from the Sardinian samples (Fig. 5B).

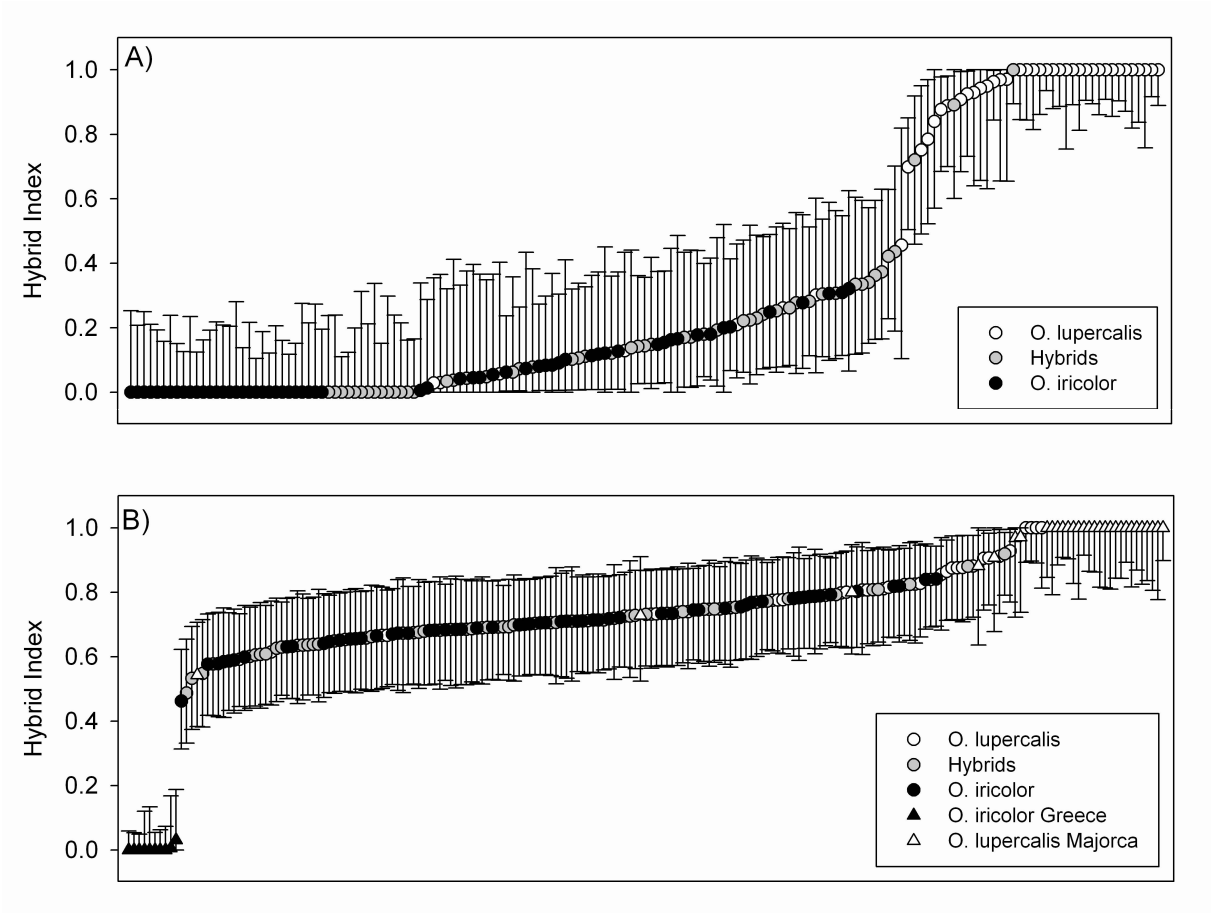

Figure 5—. A) Hybrid indices varying from 0=*O. iricolor* to 1=*O. lupercalis* for all Sardinian samples. B) Hybrid indices varying from 0=*O. iricolor* from Greece to 1=*O. lupercalis* from Majorca for all investigated species. Whiskers give 95% confidence interval.

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. This raises 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). False 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 false 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). This has previously been found for *A. nigroaenea* and *A. flavipes* which are attracted to *O. lupercalis* and *O. bilunulata*, respectively, by different bouquets of the same compounds (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 false pollination and consequently hybridization and introgression. This could be a propulsive force behind the radiation of this group.

Importance of various flower signals

We found 52 compounds in the floral odor of *O. iricolor* to release reactions in the antennae of *A. nigroaenea*. These are a few more than in previous analyses (Stökl *et al.* 2005). Improvements in EAD technique and higher amounts of compounds in the floral odor are the most probable reasons for this increase.

For *A. nigroaenea* it could be shown that the mixture of hydrocarbons, namely alkanes and alkenes, releases pseudocopulatory behavior in the males (Schiestl *et al.* 1999; Schiestl and Ayasse 2002). This results in a high selective pressure on the bouquet of hydrocarbons. Non-hydrocarbons, however, showed a flower specific variation of scent (Ayasse *et al.* 2000). Thereby plants can 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 non-hydrocarbons is not specific to the pollinator species (Stökl *et al.* 2005) and a greater difference than in the bouquet of hydrocarbons has to be expected. Interestingly, this is not the case in our results. The DFAs for hydrocarbons and non-hydrocarbons resulted in a similar proportion of correctly classified samples (57.3% and 60.5%, respectively). Previous studies (Kullenberg 1961; Schiestl *et al.* 1999) have shown that, at least in *Andrena* pollinated systems, olfactory signals are much more important for pollinator attraction than 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* showed intermediate morphological flower characters 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 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 show a complete overlap with the other samples. However, they seem to be more similar to those attractive to *A. morio* as 75% were placed with them. This is also in agreement with our behavioral observations, were more flowers of *O. iricolor* were attractive to *A. nigroaenea* than were flowers of *O. lupercalis* to *A. morio*. The finding that there is no certain type of flower odor, for example an intermediate, that attracts both pollinators, leads us to conclude, that other factors may also play a role. Previous encounters of the males with females or *Ophrys* flowers or the age of the males could influence their behavior. The different mating behaviors of both species (Ayasse *et al.* 2001) may also play an important role. The distribution in space and time of receptive females and competing males may influence the motivation of the males to copulate with a flower or not. In our observations, males of *A. nigroaenea* can often be found patrolling a rather small area in high numbers on their search for females and males engage in a scramble contest for females. In contrast, we found single males of *A. morio* searching in larger areas for females. In *A. nigroaenea* a higher competition between males may result in a lower threshold to accept an *Ophrys* flower that does not completely fit into their 'odor window'.

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 (*ssp. *archipelagi),* and *O. garganica*, whereas the species could not be separated in a microsatellite analysis. This is in contrast to our results, where 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 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, while this is not the case for populations of *O. lupercalis* from Sardinia and Majorca. Furthermore, our data show (Fig. 3, Fig. 4), that most putative hybrid plants are more similar to *O. iricolor* than to *O. lupercalis*. This suggests that *O. iricolor* has almost been replaced by an *O. iricolor x 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 x 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 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 a convergent evolution of *O. iricolor*-type morphology in the western and eastern part 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 false-pollination and hybridization between *O. iricolor* and *O. lupercalis*. A wider genetic analysis of eastern and western *O. iricolor* populations would help to 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. Here *O. iricolor* blooms from the end of February till 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 flowering period of the *Ophrys* species. Paulus (unpubl. data) found similar hybrid populations among *O. iricolor* and *O. lupercalis* in Tunisia and Malta. Until now, only *A. nigroaenea* could be found as a pollinator. If this is true, the reasons for the breakdown of the isolation barrier should be valid there too.

Smith & Ayasse (1987) and recently Vereecken *et al.* (2007) could show 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.

No hybrids between the two bee species have been found so far. We therefore postulate additional isolation mechanisms for the bees. This could be 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 Scheuchel 1997). It is possible, that this also prevents successful mating between species.

Conclusion

Our data evidently show the breakdown of the reproductive barriers between *O. lupercalis* and *O. iricolor* on Sardinia. Shifted flowering periods on Sardinia may be the main reason for the breakdown. Studies on other sympatric populations of the two species, especially in Malta and Tunisia were similar hybridization populations can be found, could help to support this hypothesis. Furthermore, factors influencing the male bee's reactions should be better investigated. Not all males react in the same way to an odor bouquet. Other factors than the floral odor may also be important for a successful pollination. False pollination and consequently hybridization seem to be more frequent in *Ophrys* than previously thought and they may have played a major role in the speciation process of several *Ophrys* species.

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Comparison of the floral odor, hybridization, and radiation of three *Andrena*-pollinated *Ophrys* species on Majorca

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Abstract—*Ophrys* orchids rely on their flower odor to attract their pollinators. At close range, in addition are visual and tactile stimuli also important. The floral odor was shown to be identical to the female sex pheromone of the pollinator and therefore attracts only males to the flowers. The males try to copulate with the flower and thereby transfer the pollen. *Andrena nigroaenea* and *A. flavipes* are attracted to flowers of *O. lupercalis and O. bilunulata* by different bouquets of the same compounds, mainly hydrocarbons. Reproductive isolation of *Ophrys* is based on the selective attraction of only a single pollinator species. Thus, attraction of a new pollinator species by changes in the bouquet of the floral odor could result in reproductive isolation and possibly speciation. AFLP analysis of *O. lupercalis, O. bilunulata*, and *O. fabrella* showed hybridization of *O. bilunulata* with both other species. In this investigation we studied and compared the pollinator attracting floral signals of *O. lupercalis, O. bilunulata*, and *O. fabrella* on Majorca using behavioral

assays, electrophysiology (GC-EAD), chemical-, and morphological analyses. Our aim was to investigate the role of scent variation in processes of speciation.

In chemical analyses and electrophysiological investigations we found 19 peaks comprising 25 compounds to release reactions in *A. fabrella* male antennae. Compounds were saturated and unsaturated hydrocarbons (alkanes and alkenes) with a chain length from 19 to 29 and non-hydrocarbons (aldehydes and esters) with a chain length from 16 to 22. Most of the compounds are also EAD-active in males of both, *A. nigroaenea* and *A. flavipes*. A comparison of the EAD-active hydrocarbons using multivariate analysis showed an overlap of the pollinator attracting scent. Flowers of *O. bilunulata* located in this overlapping area were attractive to males of *A. flavipes*, suggesting cross-pollination between species. The bouquets of EAD-active non-hydrocarbons were also specific for each species, but do also overlap. In an analysis of 17 morphological flower characters *of O. lupercalis* and *O. bilunulata* we found only minor differences between species, indicating a much lower selective pressure on flower morphology than on pollinator attracting odor. Our data support the hypothesis, that odor variation does play an important role in the reproductive isolation, hybridization and speciation of *Andrena*-pollinated *Ophrys* species.

Introduction

Sexually deceptive orchids do not reward their pollinators with nectar or pollen but trick them into pollination by mimicking a mate. The flowers imitate virgin female insects, mostly hymenoptera, in form and scent to attract males. The mislead males try to copulate with the putative female in a so-called pseudo-copulation and thereby the flowers are pollinated (Kullenberg 1961).

Pollination by sexual deception is exclusive to Orchidaceae (Dafni 1984; Ackermann 1986; Nilsson 1992). It was first described by Pouyanne (1917) and can be found in Europe (Kullenberg 1961; Paulus and Gack 1990), South Africa (Steiner *et al.* 1994), South America (van der Pijl and Dodson 1966; Singer 2002), and Australia (Peakall 1990). In Europe only the terrestrial genus *Ophrys* is pollinated by sexual deception. It has its centre of distribution around the Mediterranean basin, but can also be found in central Europe. The genus consists of more than 200 species (Delforge 2006). Pollinators are mostly bees (Andrenidae, Anthophoridae, Colletidae, Megachilidae, and Apidae), but also wasps (Sphecidae and Scoliidae) and two

beetles (Scarabaeidae) (Kullenberg 1961, 1973; Borg-Karlson 1990; Paulus and Gack 1990; Paulus 2006).

Crucial for a successful pollinator attraction is the floral odor, which is identical to the sex-pheromone of virgin females of the pollinator species (Schiestl *et al.* 1999; Ayasse *et al.* 2003; Mant *et al.* 2005a). The pollinator attracting scent can consist of a few or even a single chemical substance, as it is in the case of the attraction of *Campsoscolia ciliata* males by *Ophrys speculum* (Ayasse *et al.* 2003). In other systems the floral odor is a complex bouquet of several chemical compounds (reviewed in Ayasse 2006). Males of the *Andrena nigroaenea* are attracted to flowers of *O. sphegodes* by a mixture of saturated and unsaturated hydrocarbons (Schiestl *et al.* 1999). The same hydrocarbons, but in different amounts attract males of *A. flavipes* to flowers of *O. bilunulata* (Schiestl and Ayasse 2002). Stökl *et al.* (2005) increased the number of investigated species and analyzed the odor bouquets of several allopatric and sympatric species of the *Ophrys fusca* group, pollinated by either *A. nigroaenea* or *A. flavipes*. They found that *Ophrys* species with the same pollinator, independent of their phylogenetic relationship, always use the same odor compounds for pollinator attraction. Differences between the *Ophrys* species pollinated either by *A. nigroaenea* or by *A. flavipes* mainly involve different odor bouquets. In congruence with previous results (Schiestl and Ayasse 2002), Stökl *et al.* (2005) confirmed that alkanes and alkenes are most important for pollinator attraction. In recent investigations we got similar results in a comparison of the sympatrically occurring orchids *O. fusca* and *O. iricolor* on Sardinia. The species-specific attraction of the pollinating bees, *A. nigroaenea* and *A. morio*, respectively, is achieved by different mixtures of the same hydrocarbons and not by different volatiles (Stökl *et al.* 2007; Stökl *et al.* in prep. a).

Beside the pollinator attracting bouquet of hydrocarbons the floral odor also contains a number of non-hydrocarbons, mostly aldehydes. The bouquet of these compounds varies from flower to flower to prevent the males from learning the odor of an *Ophrys* species and to avoid their flowers in future encounters (Ayasse et al 2000).

Although the flower odor is the decisive factor for the attraction of the males, the flower morphology does also play an important role. Morphological structures guide males after landing on a flower into the correct position to take off or deposit the pollinia and therefore could function as a pre-mating isolation barrier.

As the female sex pheromones of the pollinators are species specific, an *Ophrys* species can normally attract only one pollinator species. Floral scent was shown to be the most important mechanism that is responsible for the reproductive isolation between *Ophrys* species (Bergström 1978; Ehrendorfer 1980; Paulus and Gack 1990; Stökl *et al.* 2005; Ayasse 2006). However, this mechanism does not provide a complete isolation, as hybrids and hybrid swarms between *Ophrys* species can be found often (Stebbins and Ferlan 1956; Danesch *et al.* 1975; Ehrendorfer 1980).

Floral scent and reproductive isolation may also play an important role in the evolution and radiation of *Ophrys*. If the floral scent of a hybrid plant by chance resembles the female sex-pheromone of a different and new pollinator species, the hybrid swarm would be reproductively isolated from its parental species. Subsequently, a new species of hybrid origin could evolve. However, a new species could also arise from a mutant that attracts a new pollinator species.

On Majorca there are three sympatrically occurring species of the *O. fusca* group (Paulus 2001). We already investigated several populations of the sympatrically occurring species *Ophrys lupercalis*, pollinated by *A. nigroaenea*, with a flowering season from December to March, and *O. bilunulata*, pollinated by *A. flavipes*, flowering from February to the middle of April (Paulus 2001; Schiestl and Ayasse 2002). The third species is *O. fabrella*, pollinated by *A. fabrella*, flowering from the end of March to the end of April (Delforge 2006). It is likely to be the sister species of *O. bilunulata*. There is an overlap in the flowering periods of *O. lupercalis* and *O. bilunulata* on one side and *O. bilunulata* and *O. fabrella* on the other side. Usually most specimen of all three species can be distinguished by morphological traits (Delforge 2006). However, occasionally specimen with intermediate morphological characteristics between these species can be found (Ayasse, personal observation). A comparison of AFLP markers indicated gene flow occurring across species boundaries and showed hybridization of *O. bilunulata* with *O. lupercalis* and *O. fabrella* (Stökl *et al.* in prep. b).

We assume that *O. fabrella*, a species that is endemic on the Balearic Islands, may have derived from *O. bilunulata*. This hypothesis claims that mutants of *O. bilunulata* may have produced a slightly different bouquet of hydrocarbons and attracted another pollinator (*A. fabrella*).

We investigated and compare the flower morphology and the pollinator attracting scents of *O. lupercalis, O. bilunulata*, and *O. fabrella* on Majorca with the aim to study its role in processes of speciation. Thereby we addressed the following questions:

1) Is *A. fabrella* attracted by a different bouquet of the same hydrocarbons as *A. nigroaenea* and *A. flavipes*? 2) How much do the odor bouquets of *O. lupercalis, O. bilunulata*, and *O. fabrella* differ? 3) Are all plants of *O. bilunulata* attractive to its pollinators *A. flavipes*? 4) Are there differences in the flower morphology between species?

Material and Methods

Sample collection

Samples of *O. lupercalis* DEVILLERS-TERSCHUREN & DEVILLERS, *O. bilunulata* RISSO, and *O. fabrella* PAULUS & AYASSE were collected on Majorca from 1999 to 2004 (Table 1). Species were determined directly in the field accordings to species specific morphological flower characters (Delforge 2006). Flowers that showed intermediate flower morphology and could therefore not be assigned to a species were classified as hybrids. For collection of flower odor, the labellum of a flower was cut of and put in 1.5ml pentane (Sigma-Aldrich, UvaSolv) for 48 hours, afterwards removed and samples stored at -20°C until chemical analysis. For morphological measurements, whole flowers, including sepals and petals, were put in 70% ethanol.

Behavioral experiments

Flowers were collected with the stem and put in water to keep them fresh. Flowers treated that way remain attractive for approximately one week. At places were many males of *A. flavipes* were patrolling along bushes on their search for females, the collected flowers were put in the flight path of the males. If a male performed a pseudocopulation on a flower, this flower was scored as attractive. Behavioral experiments were performed near C'an Picafort, where regularly hundreds of patrolling males of *A. flavipes* were found

Table 1—Location, date, and number of samples collected from *O. lupercalis*, *O. bilunulata*, and *O. fabrella* on Majorca. [1] No GPS data available.

Species	Location	Date	N	GPS Coordinates (UTM)		
				Zone	East	North
O. lupercalis	Capdella	23.01.2004	12	31	453699	4379167
	S'Arraco	23.01.2004	25	31	446568	4381320
	Son Viguet	23.01.2004	6	31	451815	4382094
	Andratx	23.01.2004	22	31	447843	4379719
	Andratx	25.01.2004	25	31	447832	4379725
	Platjes de Mallorca	25.01.2004	11	31	457954	4369836
	Sa Grembla	25.01.2004	3	31	448610	4384257
	Algaida	26.01.2004	12	31	484891	4380053
O. bilunulata	Mirador[1]	24.03.1999	8			
	Cala Agulla	03.03.2003	8	31	538733	4396284
	Son Severa	03.03.2003	12	31	530328	4385325
	Capdella	04.03.2003	5	31	453748	4379194
	Algaida	05.03.2003	18	31	484624	4380236
	S'Aranjassa	05.03.2003	56	31	483599	4375537
	C'an Picafort North	06.03.2003	7	31	512076	4402574
	C'an Picafort West	07.03.2003	12	31	511376	4401667
	C'an Picafort South	07.03.2003	8	31	514324	4399737
	Cala Figuera	09.03.2003	8	31	457914	4368948
O. lupercalis x bilunulata	Capdella	04.03.2003	1	31	453748	4379194
	Algaida	05.03.2003	2	31	484624	4380236
O. fabrella	C'an Picafort South[1]	27.03.1999	7			
	C'an Picafort South[1]	07.04.2000	5			
	C'an Picafort South[1]	16.04.2002	16			
	C'an Picafort South[1]	17.04.2002	2			
	C'an Picafort South[1]	20.04.2002	3			
	S'Aranjassa[1]	08.04.2000	1			
	S'Aranjassa[1]	17.04.2002	19			

Electrophysiology

To identify those compounds in the complex flower odor that can be perceived by the bees, we performed gas-chromatography coupled with an electroantennographic detector (GC-EAD). For an EAD the antenna of a male bee was excised and the tip cut off. The antenna was then mounted between two glas-electrodes filled with insect ringer (NaCl, KCl, CaCl). The Ag/AgCl wires in the glas-electrodes were connected via an amplifier (Syntech, Kirchzarten, Germany) to a PC running a EAD recording software (Syntech). One µl of the *Ophrys* odor sample was injected splitless into a HP6890 gas-chromatograph (Agilent Technologies, Palo Alto, USA) at 50°C oven temperature. After one minute the split valve was opened at the oven temperature increased at 10°C min^{-1} to 310°C. The GC was equipped with a DB5 capillary column (30m, 0.25mm i.d.). Helium was used as carrier gas (1ml min^{-1}

constant flow). At the end of the column, the carrier gas flow was split (1:1) and one part directed to the FID, the other part lead into a humidified air flow, that was directed over the insects antenna. Signals of the FID and EAD were recorded simultaneously by the EAD software.

Chemical analysis

Prior to analysis all samples were concentrated to 100 µl and 1 µg octadecane was added as internal standard. Samples were analyzed on a Thermo Trace gas chromatograph (Thermo Electron, Waltham, USA) equipped with a DB5 capillary column (30m, 0.25mm i.d., J&W) and a FID. Helium was used as carrier gas (1.5 ml min^{-1} constant flow). 1µl of the sample was injected splitless at 50°C. After one minute the split valve was opened and the oven temperature increased at 4°C min-1 to 310°C.

In all so far investigated *Ophrys* pollination systems, the electrophysiological active (EAD-active) compounds also were the behavioral active compounds (Schiestl *et al.* 1999; Ayasse *et al.* 2003; Mant *et al.* 2005a). The EAD-active compounds in the pollination systems of *O. lupercalis*/*A. nigroaenea* and *O. bilunulata*/*A. flavipes* are known from previous studies (Schiestl *et al.* 1999; Schiestl and Ayasse 2002; Stökl *et al.* 2005).

Structure elucidation

All samples were analyzed using a gas chromatograph series 8000 linked to a Fisons MD 800 mass spectrometer (Fisons Instruments, Ismaning, Germany); 70 eV mass spectra were taken in EI mode. Helium served as carrier gas. Separations were performed using a 30 m CP8912 VF-1 MS (Varian, Darmstadt, Germany) fused silica column (i.d. 0.25 mm; film thickness 0.25 µm). The temperature was initially kept at 60°C for 5 min. and then increased by 10°C per min to 300°C. Identification of compounds was carried out by comparison of mass spectra and retention times of natural products with corresponding data of synthetic reference samples. Double bond positions in alkenes were determined by investigation of the corresponding dimethyl disulfide adducts (DMDS) (Buser *et al.* 1983). Structures suggested by literature data (McLafferty and Stauffer 1989) were verified by independent

syntheses: aldehydes were obtained by oxidation of the corresponding alcohols, while esters were prepared from the acid chlorides and appropriate alcohols according to laboratory standard. Syntheses of alkenes were carried out via the corresponding alkynes (Brandsma 1988) and Lindlar-hydrogenation. Structure assignments were based on coinjection of natural extracts and synthetic reference compounds.

Morphometric analyses

Flowers stored in ethanol were photographed under a binocular microscope with a digital camera. Sixteen flower characters (Fig. 1) were measured from these photographs using 'Analysis' software (Soft Imaging Systems, Münster, Germany).

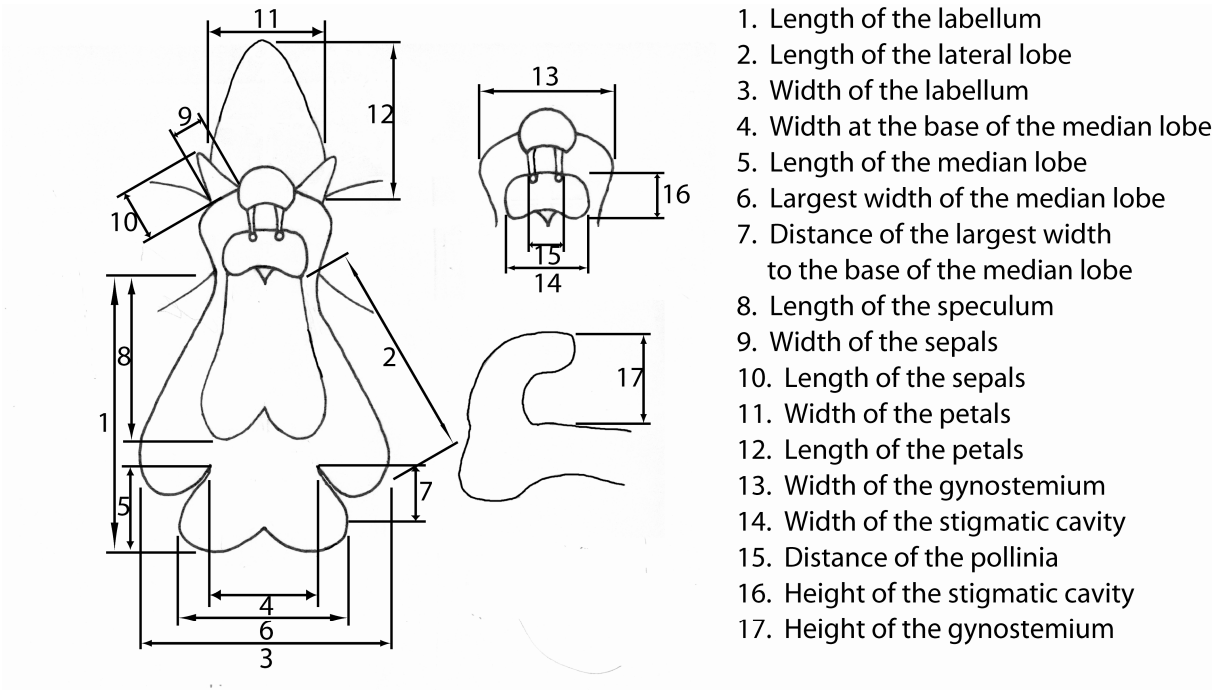

Figure 1—Morphological flower characters measured from ethanol preserved flowers.

Statistical Analysis

Analysis of floral odor was done separately for hydrocarbons and none-hydrocarbons. First, relative amounts of the EAD-active compounds were calculated. A comparison of single compounds between species was done with the Mann-Whitney U-test. For a multivariate analysis data were arc-sinus transformed and a

principal component analyses was performed to calculate principal components (PC). Resulting principal components with an eigenvalue above one were used in a discriminant function analysis (DFA). The factor loadings after varimax rotation and the standardized discriminant function coefficients were used to assess the importance of individual compounds. We considered a compound to be important, if the factor loadings above 0.5. Classification by the discriminant functions was done using the leave-one-out method. The same analysis was used for morphological data. For all calculations we used SPSS 13 (SPSS GmbH, Munich, Germany).

Results

Flower attractiveness

In 2003 we were able to test 57 flowers of *O. bilunulata* for their attractiveness to males of *A. flavipes*. Of these, 55 flowers (96.5%) were tested positively and released pseudocopulatory behavior in the males. Two flowers (3.5%) were not attractive to the males. The other flowers collected were not tested for their attractiveness to *Andrena* males

Electrophysiology

In the GC-EAD analysis 19 peaks comprising 25 chemical compounds in flower extracts of *O. fabrella* released reactions in the antennae of *A. fabrella* males (Fig. 2, Table 2). The EAD-active compounds were saturated and unsaturated hydrocarbons (alkanes and alkenes) with a chain length from 19 to 29 and non-hydrocarbons (aldehydes, ester) with a chain length from 16 to 22.

Floral odor

All compounds, that were EAD-active in at least one of the pollinator species, could be found in all three investigated *Ophrys* species (Table 2). Most of the compounds differed significantly between species.

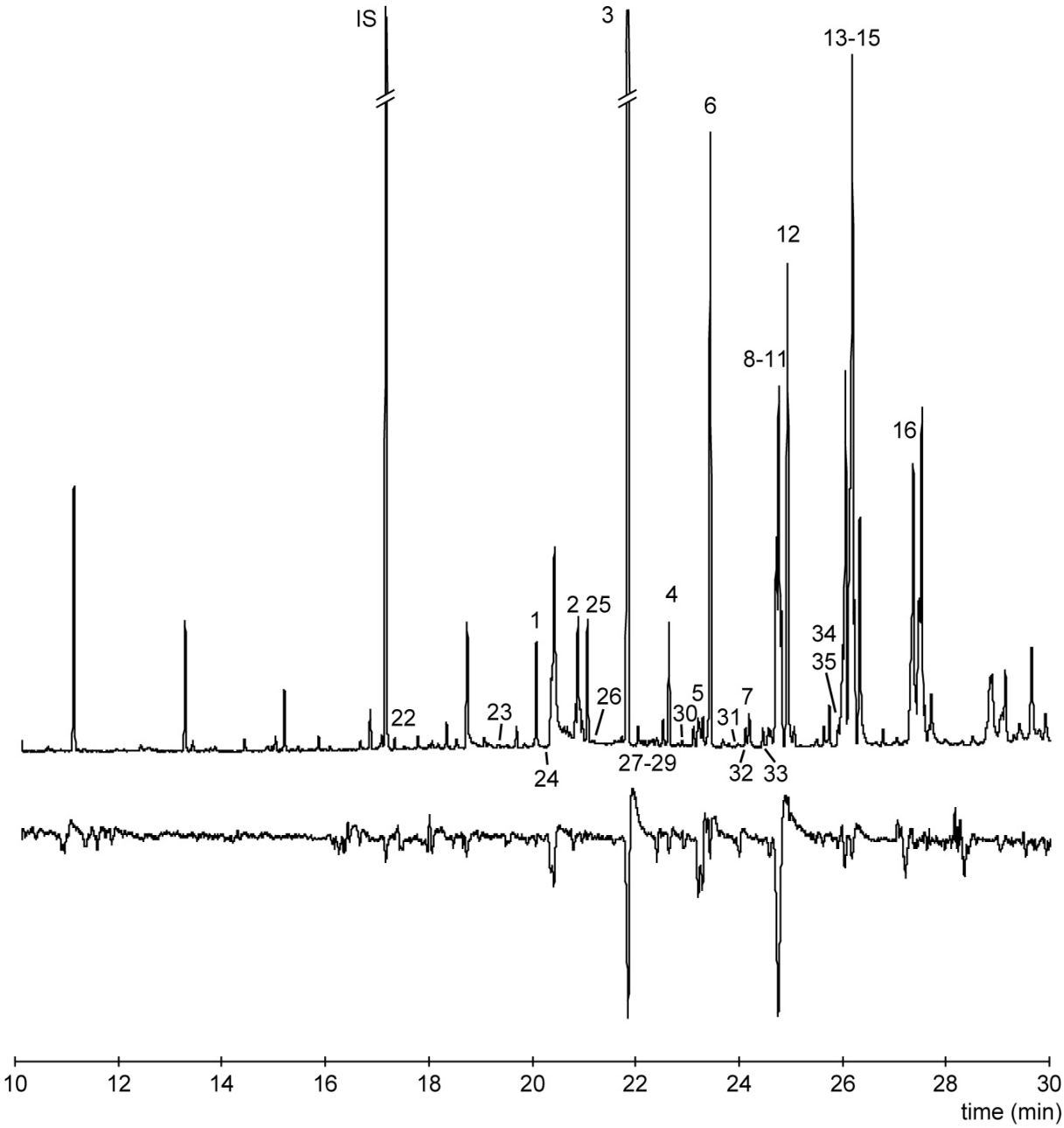

Figure 2—GC-EAD active compounds in *O. fabrella* floral odor using *A. fabrella* male antenna. Peaks are numbered according to table 2. IS=internal standard.

A principal component analysis based on the relative amounts of 23 hydrocarbons resulted in 5 principal components with an eigenvalue above one explaining 76.1% of the total variance. In the first PC alkanes with a chain length of 21, 22, 23, 24, (*Z*)-13/(*Z*)-12/(*Z*)-11-C27, and (*Z*)-14/(*Z*)-13/(*Z*)-12(*Z*)-11-C29 had the highest factor loadings. PC two had the highest factor loadings for pentacosane and alkandienes with a chain length of 29 and 31. A discriminant function analysis resulted in 3 significant functions (F1: 77.6%, χ^2=535.9, df=15, p<0.001; F2: 19.2%, χ^2 =169.8, df=8, p<0.001; F3: 3.2%, χ^2=29.2, df=3, p<0.001). The first function, explaining

77.6% of variance, had the highest function coefficient for PC4, which represented (*Z*)-12/(*Z*)-11-C25, (Z)-9-C29, and C29dien. The scatter plot of the first two functions shows a significant separation of the three species, but with overlapping areas between all of them (Fig. 3A). Samples of *O. bilunulata* and *O. lupercalis* were correctly classified at 89.7% and 97.6%, respectively. 74.2% of the samples of *O. fabrella* were correctly classified, while 12.9% each were classified to *O. lupercalis* and *O. bilunulata*. Overall, 90.2% of all cases were correctly classified by the DFA. The putative hybrid individuals did not form a distinct cluster in this analysis.

To asses the variation within species, we calculated the distance for each sample to the group centre of the corresponding species in the DFA. The median distance to the group centre was 1.31 for *O. bilunulata*, 0.95 for *O. lupercalis*, and 1.14 for *O. fabrella*. A significant difference was only found between *O. bilunulata* and *O. lupercalis* (Mann-Whitney U-test, Bonferroni correction, $p<0.001$).

To test whether the positively tested flowers form a distinct group within *O. bilunulata*, we performed a second DFA based on the hydrocarbons. In this analysis, samples were grouped by their attractiveness to males of *A. flavipes* (three groups: positive, negative, not tested). The resulting discriminant functions were not significant (F1: 86.6%, $\chi^2=6.491$, df=12, p=0.889, F2: 13.4%, $\chi^2=0.884$, df=5, p=0.971). Thus, the positively tested samples of *O. bilunulata* are representative for all samples. We can assume that the not tested samples of *O. bilunulata* would have been attractive to males of *A. flavipes*.

The principal component analysis of 19 non-hydrocarbons resulted in 6 principal components with an eigenvalue above one, which explain 69.9% of variance. In the first PC, aldehydes with a chain length of 10, 12, 19, 20, 21, and 22 had the highest factor loadings. In the second PC, aldehydes with a chain length of 9, 11, and 18, and two unidentified compounds had the highest factor loadings. The DFA resulted in three functions (F1: 83.5%, $\chi^2=528.5$, df=18, $p<0.001$; F2: 16.2%, $\chi^2=132.9$, df=10, $p<0.001$; F3: 0.3%, $\chi^2=3.1$, df=4, p=0.541). In the first discriminant function PC1 had the highest functions coefficient. The scatter plot of function one and two shows distinct clusters for each species, with an overlap between all of them. *O. fabrella* has a greater overlap with *O. bilunulata*, than with *O. lupercalis* (Fig. 3B). This is also represented by the classification results. Samples of *O. fabrella* were correctly classified at 74.2%, 19.4% were classified with *O. bilunulata* and 6.5% with *O. lupercalis*. Samples of *O. bilunulata* and *O. lupercalis* were correctly classified at

91.2% and 95.2%, respectively. Overall, 89.2% of the samples were correctly classified.

Flower morphology

Morphometric measurements were only performed with samples of *O. lupercalis* and *O. bilunulata*. The PCA produced three PC with an Eigenvalue above one, explaining 68.1% of variance. In PC one characters 6, 9, 11, 13, 14, and 15 had factors loadings above 0.5. The second PC had the highest factor loadings for characters 1, 2, 3, 4, and 7. The DFA produced two discriminant function (F1: 87.1%, χ^2=33.5, df=6, p<0.001; F2: 12.9%, χ^2=4.7, df=2, p=0.097). Principal component one showed the highest discriminant function coefficient in discriminant function one. The scatter plot showed a complete overlap of *O. bilunulata* and *O. lupercalis* (Fig. 3C). The three investigated putative hybrids are placed outside of the cluster of the two species. Due to the low number of hybrid plants, this is not significant, as show by the classification results: 81.0% of the *O. bilunulata* samples were correctly classified and 38.6% of the *O. lupercalis* samples. No hybrid plants were correctly classified. Overall, 62.5% of all cases were correctly classified.

Discussion

EAD-active compounds

Our results showed hydrocarbons, namely alkanes and alkenes, as well as some non-hydrocarbons, to release reactions in the antennae of *A. fabrella* males. Most of those compounds are also EAD-active in males of *A. nigroaenea* and *A. flavipes* (Schiestl *et al.* 1999; Schiestl and Ayasse 2002; Stökl *et al.* 2005). In all *Ophrys* pollination systems investigated so far, the EAD-active compounds were also the behaviorally active ones. Thus, *O. fabrella* attracts its pollinator, *A. fabrella* males, with almost the same set of hydrocarbons that is used by *O. lupercalis* and *O. bilunulata* to attract *A. nigroaenea* and *A. flavipes* males. The odor shows only quantitative differences between species. Not all compounds were EAD-active in all three pollinator species (Table 2). So alternatively, differences in the set of odor-receptors

on the antennae of the *Andrena*-species could also be responsible for the specific attraction.

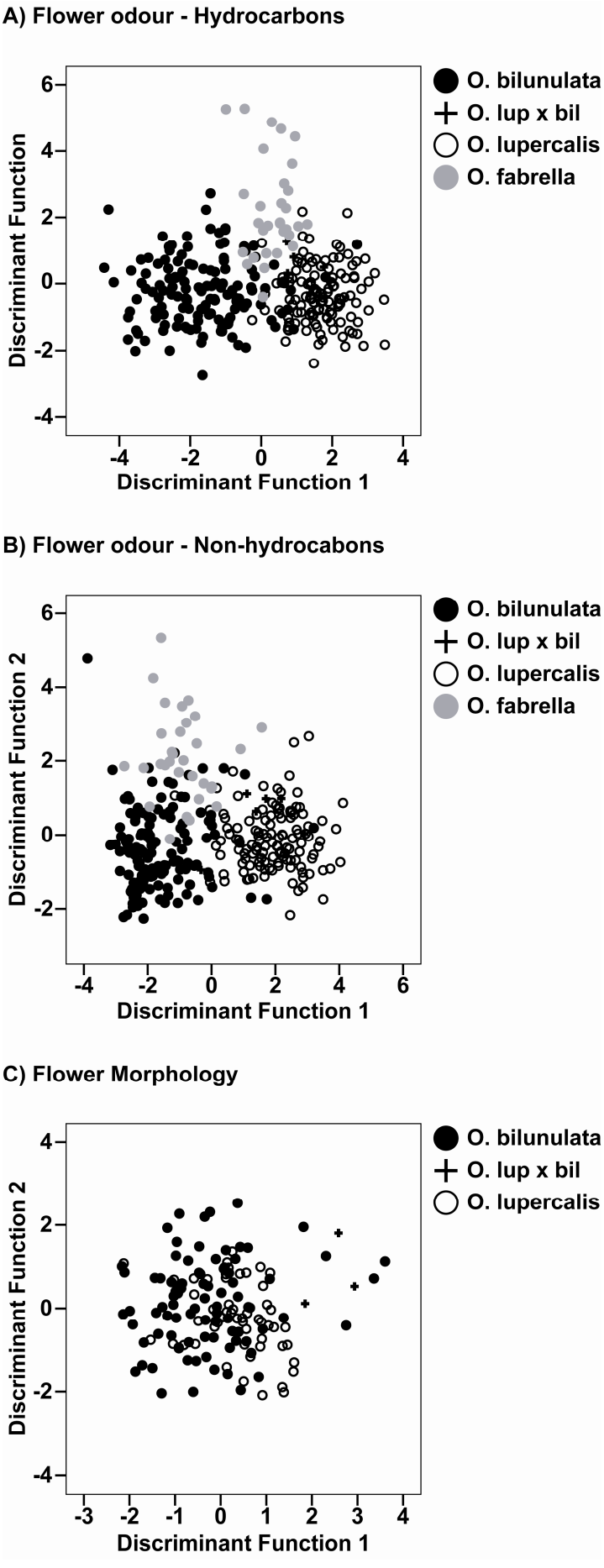

Figure 3—**Scatter plots of the first two discriminant functions of analyses of A) EAD-active hydrocarbons and B) EAD-active non-hydrocarbons in the floral odor of *O. lupercalis*, *O. bilunulata*, and *O. fabrella*. C) Scatter plots of the first two discriminant functions of an analysis of morphological flower characters of *O. lupercalis* and *O. bilunulata* (no data for *O. fabrella* available).**

Table 2—Relative amounts (mean ± SE) of the EAD-active hydrocarbons and non-hydrocarbons in the floral extracts of *O. lupercalis*, *O. bilunulata*, and *O. fabrella* and the EAD-activity in the corresponding pollinator species. [1] Compounds could not be separated with the GC-parameters used. [2] Double-bond positions unknown. [3] Letters indicated significant difference between A) *O. lupercalis* and *O. bilunulata*, B) *O. bilunulata* and *O. fabrella*, and C) *O. lupercalis* and *O. fabrella*. n.s.=no significant difference. Mann-Whitney U-test with Bonferroni-Correction, $p<0.05$. [4] EAD reactions as in Stökl *et al.* 2005. [5] Enantiomeric composition unknown.

		O. lupercalis		*O. bilunulata*		*O. fabrella*			EAD reaction in		
Hydrocarbons		mean	± SE	mean	± SE	mean	± SE	Significance[3]	*A. nigroaenea*[4]	*A. flavipes*[4]	*A. fabrella*
1	Heineicosane	1.02	0.09	0.58	0.04	2.56	0.32	A, B, C	x	x	x
2	Docosane	0.20	0.02	0.37	0.02	0.84	0.12	A, B, C	x	x	x
3	Tricosane	15.48	0.74	23.23	0.85	40.16	2.25	A, B, C	x	x	x
4	Tetracosane	1.33	0.06	2.01	0.07	2.64	0.17	A, B, C	x	x	x
5	(Z)-12/(Z)-11-Pentacosene[1]	0.28	0.04	2.19	0.15	0.85	0.13	A, B, C	x	x	x
6	Pentacosane	10.52	0.46	11.18	0.41	12.86	0.97	B	x	x	x
7	Hexacosane	0.93	0.04	0.74	0.03	0.74	0.10	A, B	x	x	
8	Heptacosadiene	0.18	0.02	0.19	0.01	0.16	0.03		x	x	x
9	(Z)-13/(Z)-12/(Z)-11-Heptacosene[1]	7.57	0.31	6.68	0.22	3.00	0.40	B, C	x	x	x
10	(Z)-9-Heptacosene	8.69	0.36	6.25	0.23	5.18	0.60	A, B	x	x	x
11	(Z)-7-Heptacosene	2.29	0.15	2.09	0.14	2.10	0.21			x	x
12	Heptacosane	10.71	0.40	12.29	0.44	11.32	0.96	A	x	x	x
13	Nonacosadiene[2]	2.45	0.13	4.83	0.16	1.84	0.23	A, C	x		
14	Nonacosadiene[2]	6.72	0.30	7.54	0.29	4.90	0.59	B, C	x	x	x
15	(Z)-14/(Z)-13/(Z)-12/(Z)-11-Nonacosene[1]	12.82	0.52	8.52	0.38	4.61	0.46	A, B, C	x		x
16	(Z)-9-Nonacosene	13.76	0.68	4.29	0.28	3.23	0.51	A, B	x	x	x
17	Hentriacontadiene[2]	5.05	0.35	7.03	0.28	3.03	0.38	A, C		x	
Non-Hydrocarbons											
18	Nonanal	12.53	0.32	12.23	0.26	8.29	0.43	B, C	x	x	
19	Decanal	1.39	0.10	4.08	0.13	1.69	0.12	A, B, C	x	x	
20	Undecanal	3.74	0.16	3.65	0.17	1.27	0.17	B, C	x	x	
21	Dodecanal	0.59	0.04	2.58	0.13	1.01	0.17	A, B, C	x	x	
22	Tridecanal	1.22	0.08	2.23	0.12	3.13	0.72	A, B	x	x	
23	Hexadecanal	1.09	0.07	2.10	0.13	1.70	0.37	A, B	x	x	x
24	Octadecanal	4.01	0.16	5.97	0.21	4.28	0.34	A, C	x	x	
25	Nonadecanal	1.40	0.24	7.03	0.34	4.36	0.51	A, B, C	x	x	
26	unknwon	12.21	0.77	15.37	0.56	8.70	1.35	A, C	x	x	

Table 2—continued

27	Eicosanal	14.96	0.56	7.45	0.20	12.98	0.84	A, C	x	x	
28	unknwon	0.98	0.46	0.28	0.02	0.38	0.06	A, B	x	x	
29	Heineicosanal	1.13	0.09	4.63	0.23	2.25	0.18	A, B, C		x	
30	unknwon	2.83	0.21	1.52	0.14	0.90	0.31	A, B, C	x	x	x
31	Docosanal	14.66	0.48	7.61	0.25	12.31	0.52	A, B, C		x	x
32	2-nonyl hexadecanoat[5]	2.31	0.22	5.16	0.53	1.73	0.58	A, B, C	x	x	x
33	unknwon	0.48	0.07	0.66	0.06	1.40	0.17	A, B, C		x	
34	Tetracosanal	7.70	0.26	7.61	0.26	11.38	0.83	B, C	x	x	
35	unknwon	7.04	0.76	4.08	0.30	13.85	1.49	A, B, C	x	x	
36	Hexacosanal	9.73	0.54	5.75	0.19	8.41	0.70	A, C		x	x

In our analysis here, a lower number of non-hydrocarbons were detected to be EAD-active in *A. fabrella* than in the other *Andrena* species investigated so far. At the time the EADs were performed (2000) only a less sensitive EAD equipment without make-up gas at the splitter was used. Supply of make up gas at the splitter results in sharper GC peaks and better reactions of the antenna (Schiestl and Marion-Poll 2002). Especially compounds that occur only in low amounts in the floral extracts may have not released a reaction then.

Cuticular hydrocarbons of insects are supposed to primarily serve as a moisture barrier against dehydration (Lockey 1988). However, in many social insects as ants, bees, wasps, and termites, cuticular hydrocarbons also form as colony specific profile, which is used for nestmate recognition (Breed 1998; Clement and Bagberes 1998; Singer *et al.* 1998; van der Meer and Morel 1998; Howard and Blomquist 2005). Cuticular hydrocarbons were also found to have a function as sex pheromone in many insects including species of the order hymenoptera. Female released male attractant hydrocarbons are known from *Formica sp*., *Lasioglossum malachurum* (Halictidae), and *Osmia rufa* (Megachilidae)(reviewed in Ayasse *et al.* 2001). In plants, hydrocarbons can be found in the cuticle as barrier against desiccation. Hydrocarbons have also been identified in the floral scent of many plant species. Alkanes and alkenes with a chain length of 23 or longer, were however almost exclusively found in *Andrena*-pollinated *Ophrys* species. Beside from *Ophrys*, they also occur in flowers of Myrsinaceae and Oleaceae (Knudsen 2006).

Species specific floral odor

Our comparison of the pollinator attracting floral volatiles by means of a discriminant function analysis showed a distinct odor bouquet for all three species. However, the species were not completely separated and there is an overlap in the odor bouquets between all three species (Fig. 3A) that suggests that cross attraction may take place.

Although most of the alkanes differed significantly in their relative amounts between all three species (Table 2), they were not important for separation of species according to a discriminant function analysis. Standardized canonical discriminant function coefficients showed (Z)-12/(Z)-11-C25, (Z)-9-C29 as well as the dienes with a chain length of 29 and 31carbons to be most important for separation of species. (Z)-9

and (*Z*)-11-Alkenes are also the dominating compounds in *A. nigroaenea*-pollinated species of the *O. sphegodes*-group (Mant *et al.* 2005b). In a further *Ophrys*-pollinator relationship, *Colletes cunicularius* is attracted by a mixture of alkanes and alkenes to flowers of *O. exaltata*. In contrast to the *Andrena*-pollinated systems, (*Z*)-7-alkenes are the key compounds for attraction and release of copulatory behavior here (Mant *et al.* 2005a).

Our results also showed a higher variation of the floral scent in *O. bilunulata* than in the other two investigated species. This can be explained by ongoing gene flow due to hybridization and introgression. *Ophrys lupercalis* and *O. fabrella* may posses genes received by hybridization of only *O. bilunulata* while the latter species received genes from both species. Only a strong selective pressure by the pollinating males of *A. flavipes* maintains the distinct floral odor of *O. bilunulata*.

Pollinator attraction and hybridization

In our behavioral experiments almost all of the flowers of *O. bilunulata* were found to be attractive to its pollinator, *A. flavipes*, independent from the exact location within the odor-space of *O. bilunulata*. Even flowers with a scent from the overlapping areas with *O. lupercalis* and *O. fabrella* attracted males of *A. flavipes*. Therefore flowers of *O. lupercalis* and *O. fabrella* which produce a floral scent that is located in the area of overlap should therefore also be attractive to males of *A. flavipes*. This results in cross attraction of pollinators between species and consequently in hybridization and introgression between species.

The probability of cross attraction is even increased since *O. bilunulata* blooms simultaneously with *O. lupercalis* in February, and simultaneously with *O. fabrella* from the mid of March until the mid of April. Consequently, the combination of overlapping floral odor and overlapping flowering periods favors hybridization and introgression. False pollination could have been one of the reasons for the overlap of the pollinator attracting scent we found in our study. However, since false pollination may cause a loss of the adaptation of a plant to its own species specific niche it should be prevented by mechanisms of isolation like morphological barriers. For example in sympatrically occurring *Ophrys* species with the same pollinator the location of the attachment of pollinia (head and tip of the abdomen) acts as a mechanical barrier (Paulus and Gack 1990).

Unfortunately, data on the attractiveness of *O. bilunulata* for males of *A. nigroaenea* or *A. fabrella* and data on the attractiveness of *O. lupercalis* and *O. fabrella* to the three *Andrena* species are lacking so far. In future investigations behavioral experiments to prove cross attraction between species should be performed.

The assumption that an overlap in the pollinator attracting odor bouquets may cause cross attraction is confirmed by studies we performed on *O. lupercalis* and *O. iricolor* on Sardinia. In chemical analyses we could show that both species attract their pollinators *A. nigroaenea* and *A. morio* with different bouquets of the same hydrocarbons (Stökl *et al.* in prep. a). The analysis of the floral odor showed an overlap of the odor bouquets between species. Behavioral experiments proved the expected cross-attraction of both pollinator species. Finally, the genetic analysis clearly showed the resulting introgression between *O. lupercalis* and *O. iricolor*.

Actually, genetic data revealed hybridization of *O. lupercalis, O. bilunulata*, and *O. fabrella* on Majorca (Stökl *et al.* in prep. b). A comparison of the species using AFLP markers proved that a gene flow between *O. bilunulata* and *O. lupercalis* or *O. fabrella* takes place. The investigated specimen of *O. bilunulata* did not show an own species specific genotype. Part of the plants of *O. bilunulata* strongly overlapped with *O. lupercalis* and another part with *O. fabrella*. This is in contrast to our results on the analysis of floral odor, where *O. bilunulata* does showed a distinct species specific odor bouquet. This indicates a very strong selective pressure by the pollinating males on the plants to maintain the correct species specific odor bouquet.

Phylogenetic origin of Ophrys fabrella

The exact phylogenetic origin of *O. fabrella* is unknown so far. As it is endemic to the Balearic Islands, it is supposed to have diverged from another species of the fusca group, namely either *O. lupercalis* or *O. bilunulata*. According to various floral characters studied, *O. fabrella* seems to be more closely related to *O. bilunulata* than to *O. lupercalis*. In our comparison of the floral volatiles, based on non-hydrocarbons, samples of *O. fabrella* were placed nearer to *O. bilunulata* than to *O. lupercalis*. In another *Andrena* pollinated *Ophrys* species, *O. sphegodes*, the bouquet of non-hydrocarbons were shown to be not involved in pollinator attraction (Ayasse *et al.* 2000) and does therefore not underlie a pollinator driven selection. In a comparison of several *Andrena* pollinated *Ophrys* species, non-hydrocarbons were found to reflect

more the phylogenetic relationship of *Ophrys* species than hydrocarbons (Stökl *et al.* 2005). A comparison using highly conserved genetic markers has to be performed in order to investigate the phylogenetic relationship between these *Ophrys* taxa.

Flower morphology

During sample collection in the field, *O. lupercalis* and *O. bilunulata* could be clearly identified based on morphological flower traits and only a very low number of flowers with differing or intermediate flower characters have been found. However, our comparison based on 17 morphological flower characters showed only minor differences between species (Fig. 3C). However, standardized canonical discriminant function coefficients showed, that the width of the gynostemium, the width of the stigmatic cavity, and the distance of the pollinia were most important for separation of species. These characters directly determine the position of the pollinator's abdomen in relation to the pollinia and the stigma and are therefore crucial for a successful pollination. Gölz and Reinhard (1990) compared morphological flower characters of *O. lupercalis* from Majorca, *O. iricolor* from Sardinia, and *O. iricolor* from Crete. In their analysis, the height of the stigmatic cavity was an important character to separate *O. lupercalis* from *O. iricolor*. In the *Ophrys* species investigated in this study, the pollinia are placed on the pollinator's abdomen. As the males' abdomen is very flexible and is intensely moved during copulation attempts, the exact size of the flower labellum is not curial for a successful pollination.

In our investigations, we did not measure certain flower characters, as the curving of the labellum at its base or the color of the speculum, although they were used for species determination in the field. If we would have included these parameters in our analyses, we probably would have received different result. Nevertheless, the low differences in flower morphology indicate a lower selective pressure on flower morphology than on floral odor.

Conclusions

Our data indicate that reproductive isolation in *Andrena* pollinated *Ophrys* species on Majorca is not complete. Overlapping odor bouquets and overlapping

flower periods probably resulted in hybridization and introgression or alternatively could be the result of gene flow between taxa. Furthermore, based on the floral traits with used in our study we cannot prove if the overlap in floral scent and morphological flower characters are the result of a recent processes of speciation or reflect ongoing gene flow by cross attraction and hybridization. Generally, attraction of the false pollinator seems to happen more often in systems were the specific attraction is based on quantitative, rather than qualitative differences in the pollinator attracting floral odor.

Our data underline that hybridization and shifts of pollinator can be a driving force in the evolution of a group, enabling various scenarios of speciation. Our investigations clearly demonstrate that *Ophrys* orchids represent is a unique model system to study processes of speciation.

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4

Genetic variation, hybridization, and introgression in co-flowering *Ophrys* species on Majorca

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Abstract—Reproductive isolation of *Ophrys* orchids is based on the selective attraction of males of a single pollinator species by mimicking the female sex-pheromone of its pollinator. The mislead males try to copulate with the flower and thereby pollinate it. Changes in the floral odor can lead to the attraction of a different pollinator, which would act as a reproductive isolation barrier. In this study we used AFLPs to investigate the population structure and rate of hybridization of three *Andrena*-pollinated *Ophrys* species, *O. lupercalis*, *O. bilunulata*, and *O. fabrella*, on Majorca. Our data showed a clear separation of *O. lupercalis* and *O. fabrella*. Plants of *O. bilunulata* did not form a common group in the AFLP analysis, but were placed with either *O. lupercalis* or *O. fabrella*. Calculation of hybrid indices proofed the hybridization of *O. bilunulata* with both other species. This is in strong contrast to the distinct floral odor and the distinct flower morphology of *O. bilunulata* we found in previous studies. Hybridization is caused by overlapping flowering periods. Our

data show, that plants of *O. bilunulata* hybridize with *O. lupercalis* and *O. fabrella*, if flowering at the same time.

Introduction

Terrestrial orchids of the genus *Ophrys* are pollinated by sexual deception of male hymenoptera. *Ophrys* flowers imitate virgin females of their pollinators to attract the males which try to mate with the supposed female in a so-called pseudocopulation (Kullenberg 1961). Thereby the flowers are pollinated. Pollination by sexual deception was first descried by Pouyanne (1917) and is exclusive to Orchidaceae. It is known from Europe (Kullenberg 1961; Paulus and Gack 1990), Africa (Steiner *et al.* 1994), South-America (Singer 2002), and Australia (Peakall 1990). In Europe it can only be found in the genus *Ophrys*, where pollinators are mostly bees, occasionally wasps and beetles (Kullenberg 1961, 1973; Borg-Karlson 1990; Paulus and Gack 1990).

Ophrys flowers mimic their pollinators' females in shape, color and scent. While the flowers only vaguely resemble the females in shape and color, the imitation of the female sex-pheromone is almost perfect (Schiestl *et al.* 1999; Ayasse *et al.* 2003; Mant *et al.* 2005a). *Andrena nigroaenea* and *A. flavipes* are attracted by a mixture of hydrocarbons, namely alkanes and alkenes, to the flowers (Schiestl and Ayasse 2002). Furthermore, it was shown that *Ophrys* species with the same pollinator independent of their phylogenetic relationship use the same compounds in very similar composition for male attraction (Stökl *et al.* 2005).

Due to the highly specialized floral signals, an *Ophrys* species normally attracts only one pollinator species (Ayasse 2006). This selective attraction of pollinators acts as the main reproductive isolation mechanism between the mostly crossable *Ophrys* species (Paulus and Gack 1990; Ehrendorfer 1980). However, the selective attraction does not seem to be perfect, as hybrids between *Ophrys* species can be frequently found (Stebbins and Ferlan 1956; Danesch and Danesch 1972; Danesch *et al.* 1975; Ehrendorfer 1980). In *Ophrys* orchids, odor changes as a result of genetic mutations, negative frequency dependent selection (Ayasse et al 2000) or hybridization can be the driving force for speciation, since they may become the cause for reproductive isolation between the interfertile species (Ehrendorfer 1980; Paulus & Gack 1990). Alternatively, species-specific differences in floral odor may be a by-product of a

speciation process, indicating selection for attraction of a different pollinator after reproductive isolation has been established (Grant 1994).

Genetic markers are particularly successful for hybrid detection and their genetic characterization, as well as for the study of introgressive phenomena (Rieseberg & Ellstrand 1993), and have been used in several orchids (Rossi *et al.* 1992; Steinbrück *et al.* 1986). AFLPs have been successfully used in studies on population and species level (e.g. Hedren *et al.* 2001) and also to examine hybridization and the process of speciation (Wilding *et al.* 2001).

Genetic studies on *O. sphegodes* populations could show that reproductive isolation between sympatric species is not complete and that gene flow across species boundaries does occur (Soliva and Widmer 2003). Mant *et al.* (2005b) combined genetic data with an analysis of floral odor of *O. sphegodes* and *O. exaltata* in Italy. Although the floral odor was highly differentiated, genetic differences within and between populations were very low. Stökl *et al.* (2007, in prep. a) investigated the floral odor, pollinator attractiveness, and the genetic variation of *O. iricolor*, *O. lupercalis*, and their hybrids on Sardinia. They could show cross-attraction of pollinators between species, which results in hybridization and introgression between species. Due to the high rate of hybridization, *O. iricolor* has already been displaced by an *O. iricolor-lupercalis* mix-population. The same has to be expected for the remaining plants of "pure" *O. lupercalis*. All these data indicate the important role of pollination and pollinator attraction in processes of speciation and radiation of this genus. Pollinator shift may play an important role in the radiation of *Ophrys* species.

Stökl *et al.* (in prep. b) compared the pollinator attracting scent of the sympatrically occurring *Ophrys* species *O. lupercalis*, *O. bilunulata*, and *O. fabrella*, which are pollinated by different *Andrena* species, on Majorca. They found that males of *A. nigroaenea*, *A. flavipes*, and *A. fabrella*, the pollinator species, are attracted to the flowers by different bouquets of almost the same set of hydrocarbons (Schiestl and Ayasse 2002; Stökl *et al.* in prep. b). Although those species do not share all compounds in the floral odor, they have most compounds in common. Furthermore, Stökl *et al.* (in prep. b) found an overlap in the pollinator attracting scent between all three species. Behavioral experiments with flowers of *O. bilunulata* that were found to posses an odor from this zone of overlap were attractive to males of its own pollinator *A. flavipes*. Individuals of *O. lupercalis* and *O. fabrella* that were also placed in the zone of overlap therefore posses a very similar odor to the plants of

O. bilunulata tested. Although not tested in a bioassay, we can assume those plants to be attractive to males of *A. flavipes*. False pollinator and hybridization have to be expected as *O. bilunulata* overlaps in its flowering period with both other species.

In this study we used Amplified Fragment Length Polymorphism (AFLP) as a neutral genetic marker to study populations of *O. lupercalis*, *O. bilunulata*, and *O. fabrella* on Majorca. Our aim was to investigate the genetic structure of the populations and to asses the rate of hybridization and introgression between species.

Material and Methods

Investigated species

On Majorca three species pollinated by *Andrena* occur sympatrically (Delforge 2006): *Ophrys lupercalis* DEVILLERS-TERSCHUREN & DEVILLERS, which is widely distributed in the central and western Mediterranean. It is pollinated by *Andrena nigroaenea* KIRBY 1802 and has the largest flowers of the three species. *Ophrys bilunulata* RISSO is pollinated by *A. flavipes* PANZER 1799 and has a similar area of distribution as *O. lupercalis*, but is missing on Corsica and Sardinia. The third species, *O. fabrella* PAULUS & AYASSE, pollinated by *A. fabrella* PEREZ 1903, is endemic to the Balearic Islands. It is thought to have derived from either *O. lupercalis* or *O. bilunulata*, the two most closely related *Ophrys* species on Majorca. Morphological floral characters, the late flowering period, and an analysis of selectively neutral floral odor compounds indicate a closer relatedness to *O. bilunulata*, than to *O. lupercalis* (Stökl *et al.* in prep. b). The current hypothesis is, that *O. fabrella* evolved from late flowering *O. bilunulata* plants, which recruited a new pollinator species, *A. fabrella*.

On Majorca, this three species bloom consecutively. *O. lupercalis* blooms from end of December to March. O bilunulata has a flowering period from February to the mid of April. *O. fabrella* is flowering from the end of March to the end of April.

Sample collection

Samples of *O. lupercalis*, *O. bilunulata*, and *O. fabrella* were collected from 15 populations on Majorca from 1999 to 2004 (Table 1). Species were determined directly in the field by species specific morphological floral characters (Delforge 2006). Small pieces of leaves were put in plastic bags containing silica gel (Merck, Darmstadt, Germany).

Genetic Analyses

Amplified Fragment Length Polymorphism (AFLP) was used to investigate the genetic structure of the populations. 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: EcoRI+A, MseI+CT. Selective PCR was done with primers with 3 and 4 selective bases. EcoRI-Primer were fluorescently labelled at the 5'-end with 6-FAM, HEX and NED. Following primer pairs were used: EcoRI-ACA and MseI-CTGA, EcoRI-AGG and MseI-CTAG, EcoRI-AGC and MseI-CTCG.

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

Statistical analysis

AFLP data were analyzed with FAMD 1.108 (Schlüter and Harris 2006). Principal Coordinate Analysis (PCA) was based on a pairwise matrix of Jaccard's similarity index. Φ_{ST} is an analogous to F_{ST} when data are binary. Hybrid indices were calculated with Hindex (Buerkle 2005). Hybrid indices estimate the genetic contribution of hybridizing species to individuals of unknown ancestry. They vary from 0 to 1, whereby values of 0 and 1 represent the parental species. Calculation of hybrid indices was done between *O. bilunulata* and *O. lupercalis*, *O. bilunulata* and *O. fabrella*, and for *O. bilunulata* with *O. lupercalis* and *O. fabrella* as parental species.

The correlation of hybrid index and sampling date was measured using Spearman's correlation coefficient. Mean hybrid indices between populations of *O. bilunulata* were compared using the Mann-Whitney U-test. For correction of ties we used the method by Benjamini and Hochberg (1995). Correlations and U-test were calculated with SPSS 13 (SPSS, München, Germany).

Table 1—Location, date, and number of samples collected from *O. lupercalis, O. bilunulata*, and *O. fabrella* on Majorca. [1] No GPS data available.

Species	Location	Date	N	GPS Coordinates (UTM)		
				Zone	East	North
O. lupercalis	Capdella	23.01.2004	12	31	453699	4379167
	S'Arraco	23.01.2004	25	31	446568	4381320
	Son Viguet	23.01.2004	6	31	451815	4382094
	Andratx	23.01.2004	22	31	447843	4379719
	Andratx	25.01.2004	25	31	447832	4379725
	Platjes de Mallorca	25.01.2004	11	31	457954	4369836
	Sa Grembla	25.01.2004	3	31	448610	4384257
	Algaida	26.01.2004	12	31	484891	4380053
O. bilunulata	Mirador[1]	24.03.1999	8			
	Cala Agulla	03.03.2003	8	31	538733	4396284
	Son Severa	03.03.2003	12	31	530328	4385325
	Capdella	04.03.2003	5	31	453748	4379194
	Algaida	05.03.2003	18	31	484624	4380236
	S'Aranjassa	05.03.2003	56	31	483599	4375537
	C'an Picafort North	06.03.2003	7	31	512076	4402574
	C'an Picafort West	07.03.2003	12	31	511376	4401667
	C'an Picafort South	07.03.2003	8	31	514324	4399737
	Cala Figuera	09.03.2003	8	31	457914	4368948
O. lupercalis x bilunulata	Capdella	04.03.2003	1	31	453748	4379194
	Algaida	05.03.2003	2	31	484624	4380236
O. fabrella	C'an Picafort South[1]	27.03.1999	7			
	C'an Picafort South[1]	07.04.2000	5			
	C'an Picafort South[1]	16.04.2002	16			
	C'an Picafort South[1]	17.04.2002	2			
	C'an Picafort South[1]	20.04.2002	3			
	S'Aranjassa[1]	08.04.2000	1			
	S'Aranjassa[1]	17.04.2002	19			

Results

Sample collection

We collected 314 samples from *O. lupercalis*, *O. bilunulata*, and *O. fabrella* on Majorca (Table 1). The number of flowers with intermediate flower morphology was

very low. In 2003, six putative hybrids between *O. lupercalis* and *O. bilunulata* were found, of which three could be successfully analyzed.

Genetic analysis

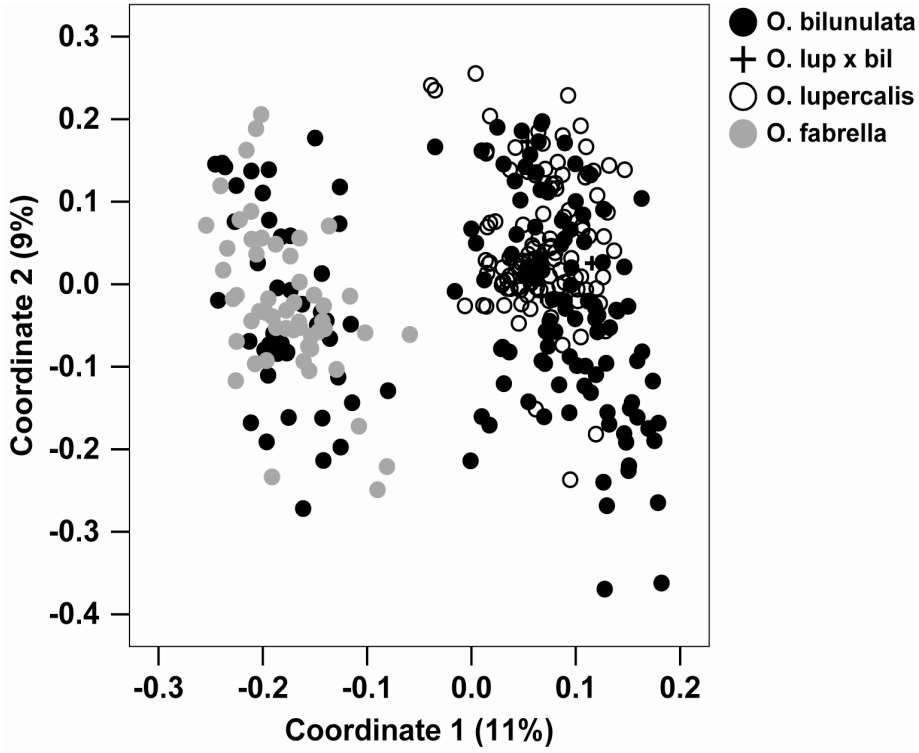
We analyzed 146 AFLP bands from three primer combinations in 315 samples. The number of polymorphic bands was 145 in *O. bilunulata*, 144 in *O. lupercalis*, 68 in the putative hybrids, and 125 in *O. fabrella*. We found no private bands in *O. fabrella* and hybrids, and one in each, *O. bilunulata* and *O. lupercalis*.

Principal coordinate analysis showed a complete separation of *O. lupercalis* and *O. fabrella* (Coordinate 1: 10.9%; Coord. 2: 8.5%; Coord. 3: 7.8% of variance). The samples of *O. bilunulata* did not form a distinct cluster, but were placed with either *O. lupercalis* or *O. fabrella* (Fig. 1). From 143 samples of *O. bilunulata*, 99 were placed in the cluster of *O. lupercalis* and 44 in the cluster of *O. fabrella*. Samples of *O. bilunulata* from the same population clustered almost always together. There is no overlap between these two clusters. Putative hybrid plants were placed in the cluster of *O. lupercalis*.

Hybrid indices clearly showed a high rate of introgression between species (Fig. 2A, B). In both analyses, *O. bilunulata* – *O. lupercalis* and *O. bilunulata* – *O. fabrella*, the transition between species was complete without a gap. Furthermore, was the hybrid index of *O. bilunulata* correlated with the sampling date (*O. bilunulata* – *O. lupercalis*: $R=-0.275$, $p<0.001$; *O. bilunulata* – *O. fabrella*: $R=0.299$, $p<0.001$). So plants of *O. bilunulata* that flower earlier in the year were more similar to *O. lupercalis* than later blooming specimen. Late flowering plants of *O. bilunulata* were more similar to *O. fabrella*.

To analyze the rate of introgression within different populations of *O. bilunulata* on Majorca, we calculated a hybrid index for *O. bilunulata* between *O. lupercalis* and *O. fabrella*. All plants within a population, except some outliers, were either similar to *O. lupercalis* or to *O. fabrella*.

Figure 1—Scatter plot of the first two axes of a principal coordinate analysis based on AFLP data (Coordinate 1: 10.9%; Coordinate 2: 8.5% of variance).



Discussion

Hybridization between species

Hybrids have been frequently described in Mediterranean orchid genera, as for example in *Orchis* or *Anacamptis* (Pellegrino *et al.* 2000, 2005). Hybrids between *Anacamptis morio* and *A. papilionacea* do not produce fertile seed, thus no gene flow across species boundaries occurs (Moccia *et al.* 2007). In contrast, no post-pollination reproductive barriers seem to have evolved in *Ophrys*. Our data clearly show hybridization and introgression of *O. bilunulata* with both, *O. lupercalis* and *O. fabrella*. Hybrid indices showed a complete transition between *O. bilunulata* and *O. lupercalis* as well as *O. bilunulata* and *O. fabrella*, and all types of intermediate plants can be found. There is a comparable situation on Sardinia, where hybrid swarms between *O. lupercalis* and *O. iricolor* have been observed. In behavioral experiments, flowers of both parental species were attractive to the pollinators of both species, *A. nigroaenea* and *A. morio* (Paulus and Gack 1995; Stökl *et al.* in prep. a). This results in a

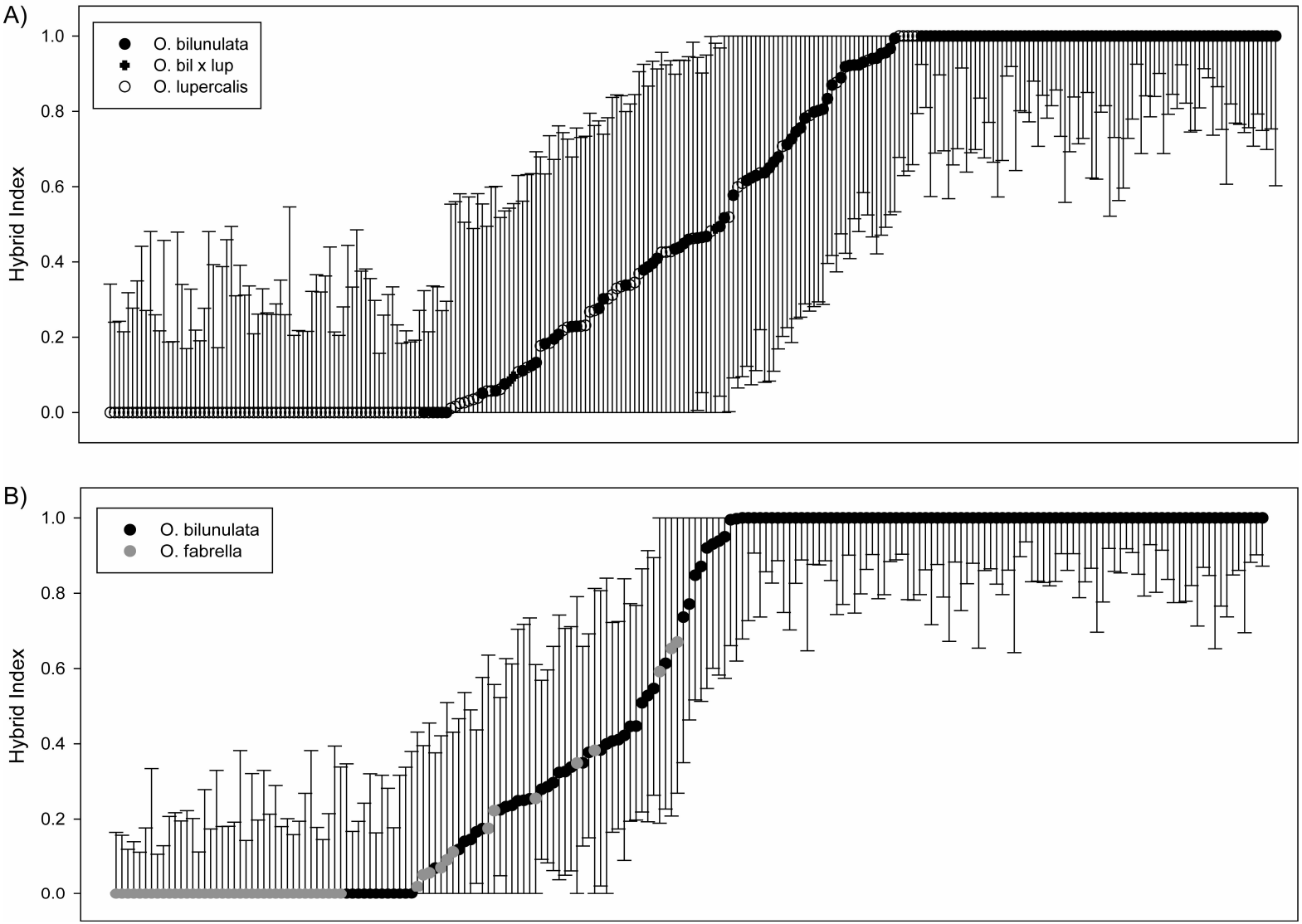
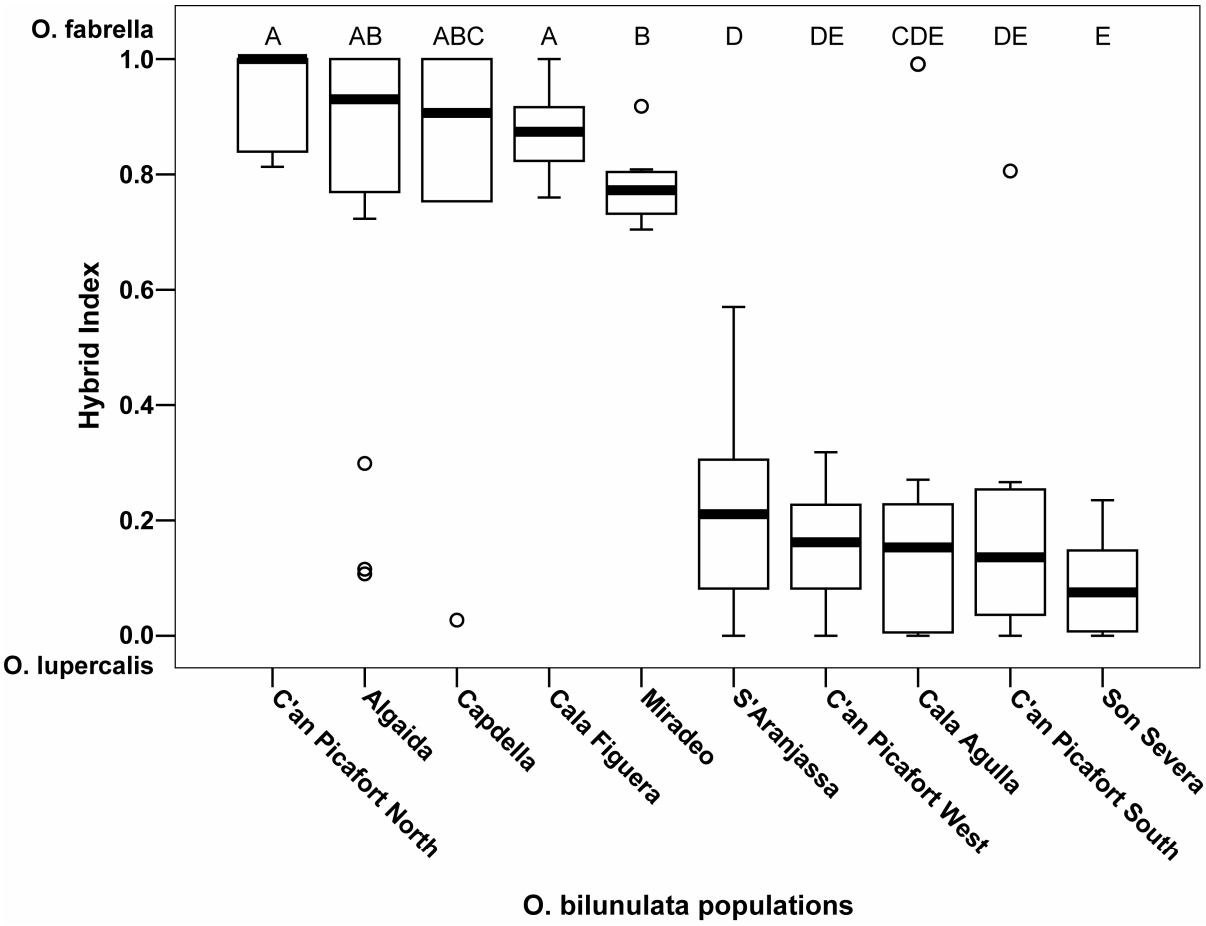


Figure 2—Hybrid index calculated between A) *O. bilunulata* and *O. lupercalis,* B) *O. bilunulata* and *O. fabrella.* Whiskers give 95% confidence interval.

high rate of hybridization and introgression between the two species. Actually, all plants of *O. iricolor* are affected by the introgression. Unaffected or pure plants of *O. lupercalis* can still be found, but only in a very low number (Stökl *et al.* in prep. a).

Figure 3—Box plots of the hybrid indices in different populations of *O. bilunulata.* Hybrid indices were calculated for *O. bilunulata* between *O. lupercalis* and *O. fabrella*. Different letters indicate significant difference between the populations (Mann-Whitney U-test with Benjamini-Hochberg correction, $p<0.05$). Circles mark outliers.



Gene flow between species has also been revealed in other *Ophrys* species. Soliva and Widmer (2003) used microsatellites to investigate several species of the *O. sphegodes* group. They report lower genetic differences between geographically distant populations of the same species, than between sympatric populations of different species. However, the number of actual hybrids seems to be rather low. A later study confirmed the data of Soliva and Widmer (2003). Mant *et al.* (2005b) found very low genetic differences between three species of the *O. sphegodes* group. Interestingly, the same specimen showed clear differences in the species-specific pollinator attracting scent.

Schlüter *et al.* (2007) found a similar situation in the *O. omegaifera* complex in the Aegean. In an AFLP-analysis of several sympatrically occurring species, *O. sitiaca* was genetically indistinguishable from *O. omegaifera*, while the other investigated species were well separated. They conclude that ongoing gene flow between species is the most likely explanation for the detected mixing of species. Whether gene flow is mediated by *Anthophora atroalba* or *Andrena nigroaenea*, the pollinator species of *O. omegaifera* and *O. sitiaca*, respectively, or by an third, unknown pollinator species is unclear.

Causes of hybridization

All three *Ophrys* species we investigated attract their pollinators with different bouquets of almost the same hydrocarbons (Stökl *et al.* in prep. b). A comparison of the odor bouquets showed a high intraspecific variation and an overlap between all species, causing cross attraction of pollinators. Intraspecific variation of the floral odor can be caused by genetic mutations or negative frequency dependent selection by the pollinator, as shown for *O. sphegodes* (Ayasse *et al.* 2000).

An overlap in the floral odor alone does not necessarily lead to hybridization, if the plants of the two species do not flower sympatrically at the same time. However, the flowering period of *O. bilunulata* does overlap with that of *O. lupercalis* in February and with that of *O. fabrella* from the end of March to the mid of April. An overlap of the flowering period and the high similarity of the odor bouquets might therefore have been the reasons for hybridization. No signs of hybridization of *O. lupercalis* and *O. fabrella* have been found. Flowering periods of these two species are well separated and do not overlap, which successfully prevents hybridization between them.

Genetic structure of Ophrys bilunulata populations

A comparison of the hybrid indices in the investigated populations of *O. bilunulata* that occur in sympatry with either *O. lupercalis* or with *O. fabrella* showed two groups.of genotypes (Fig. 3). The question arises why we did not find a unique genotype in the *O. bilunulata* populations investigated. This can be explained by a sympatric occurrence of the corresponding species, *O. lupercalis* and *O. fabrella*, in the

studied populations on one hand and a limited dispersal of pollen by short flight distances of the *Andrena* males on the other hand. Dispersal of pollen is limited by the flight distance of the pollinating insects. In a mark and recapture study on males of *Colletes cunicularius*, the pollinator of four *Ophrys* species, males were recaptured at maximum 50m away from the marking site (Peakall and Schiestl 2004). Although we do not know the maximum flight distances of the *Andrena* males involved in the pollination of our *Ophrys* species we can assume that they are not able to transfer pollen of one genotype that occurs in one population to the other genotype that can be found in another population over a distance of many kilometres.

Our results show a correlation of the hybrid index of *O. bilunulata* with the date of sampling. Flowers collected early in the flowering season were more similar to *O. lupercalis*; flowers collected late are more related to *O. fabrella*. Although, the correlation is significant, the correlation coefficients are not very high (-0.275 and 0.299). We collected our samples of *O. bilunulata* not over the whole flowering period and furthermore is the date of sampling of a plant not identical to the date of the first flowering. This could explain the low correlation coefficients obtained.

So far we do not know if both successively flowering genotypes of *O. bilunula*ta occur within the same population. More data on the sympatric occurrence of *O. lupercalis* and *O. fabrella* in the populations of *O. bilunulata*, samples of early and later flowering O. bilunulata specimen of the same populations and the maximum flight distances of *Andrena* males are needed to support one or the other hypotheses.

Species status of Ophrys bilunulata

In the PCO, samples of *O. bilunulata* did not form a separate cluster but were placed with either *O. lupercalis* or *O. fabrella*. This result raises doubts on the species identity of *O. bilunulata* on Majorca. However, *O. bilunulata* is a widespread species which has the same floral characters and the same pollinator species in all populations so far studied. Stökl *et al.* (in prep. b) found no differences in the flower morphology and in the pollinator attracting scent between the two groups. Therefore, no reproductive barrier between the two groups exists. Therefore, the current genetic structure of *O. bilunulata* can be explained by hybridization with *O. lupercalis* and *O. fabrella*. Further investigations including populations of *O. bilunulata* outside of Majorca using genetic methods should be performed.

Genotype and phenotype of O. bilunulata

In our genetic analysis, the samples of *O. bilunulata* did not from a distinct group, which could be separated from *O. lupercalis* and *O. fabrella*. This is in strong contrast to the observed phenotype of *O. bilunulata*. The species can be easily distinguished from *O. lupercalis* and *O. fabrella* by morphological flower characters in the field. Furthermore, recent investigations of Stökl *et al.* (in prep. b) showed a very similar floral odor in all plants of *O. bilunulata*, significantly different to the odor of *O. lupercalis* and *O. fabrella*. This strong discrepancy between these two data sets can only be explained by a very strong selective pressure by the pollinating males of *A. flavipes* on the floral odor of *O. bilunulata.*

Phylogenetic origin of Ophrys fabrella

We hypothesize that *O. fabrella* evolved from late flowering *O. bilunulata* plants, which recruited a new pollinator species, *A. fabrella*. This hypothesis is supported by our data. First of all, our genetic analyses showed that *O. fabrella* is more similar to *O. bilunulata* than to *O. lupercalis*. Furthermore, it showed a smaller number of polymorphic bands in the AFLP analyses in comparison to the other two species, which indicates a younger age of this species. Wider phylogenetic analyses including samples of *O. bilunulata* and *O. lupercalis* from other European populations should be performed to study the origin of *O. fabrella*.

Conclusion

In our study we could show that *O. bilunulata* hybridizes with both, *O. lupercalis* and *O. fabrella*, which was expected because of overlapping floral odor and flowering periods (Stökl *et al.* in prep. b). Furthermore, we found strong differences between the genotype and the phenotype of *O. bilunulata*, caused by a pollinator driven selection. The possible reasons for the occurrence of two different genotypes we found, however, are still unclear. A first explanation could be a recent process of radiation and an ongoing process of speciation. Alternatively, cross pollination and hybridization could also have been responsible for the genetic structures we found.

Our data emphasize the importance of pollination and hybridization for processes of speciation and radiation in *Ophrys* orchids.

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Stökl J, Twele R, Erdmann DH, Francke W, Ayasse M (2007) Comparison of the flower scent of the sexually deceptive orchid *Ophrys iricolor* and the female sex pheromone of its pollinator *Andrena morio*. Chemoecology published online

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Publications from this thesis

The present thesis is based on the following four manuscripts, which have been accepted for publication (manuscript 1) or will be submitted to scientific journals.

1. Stökl J., Twele R., Erdmann, D. H., Francke W., Ayasse M. (2007) Comparison of the flower scent of the sexually deceptive orchid *Ophrys iricolor* and the female sex pheromone of its pollinator *Andrena morio*. Chemoecology published online

2. Stökl J., Schlüter P. M., Stuessy T. F., Paulus H. F., Assum G., Ayasse M. (in prep.) Scent variation and hybridization cause the displacement of a sexually deceptive orchid species on Sardinia.

3. Stökl J., Fraberger R., Erdmann D. H., Schulz C., Francke W., Ayasse M. (in prep.) Comparison of the floral odor, hybridization and radiation of three *Andrena*-pollinated *Ophrys* species on Majorca

4. Stökl J., Schlüter P. M., Stuessy T. F., Paulus H. F., Assum G, Ayasse M. (in prep.) Genetic variation, hybridization, and introgression in co-flowering *Ophrys* species on Majorca

Contributions of co-authors

Manfred Ayasse: As my supervisor he added a lot of guidance, ideas, and valuable comments to all parts of my thesis. He is therefore co-author on all manuscripts derived from this thesis.

Philipp Schlüter, Tod Stuessy, Hannes Paulus: The AFLP markers used in this study were adapted for the investigated species in collaboration with them in Vienna. They also provided me with *Ophrys*-samples from Greece. They are co-authors on the manuscripts 2 and 4.

Günter Assum: Analysis of the AFLP band on the sequencer was done is his lab.

Raphaeleo Fraberger: He did the GC-EAD analysis with *Ophrys fabrella* and *Andrena fabrella*. He is therefore co-author on manuscript 3.

Robert Twele, Dirk Erdmann, Claudia Schulz, Wittko Francke: Wittko Francke and co-workers did the structure elucidation of the odor compounds. They are co-authors on manuscripts 1 and 4.

Part of the DNA-extractions and the morphometric measurements on the flowers were done by student assistants.

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Stökl J., Paulus H. F., Dafni A., Schulz C., Francke W., Ayasse M. (2005) Pollinator attracting odour signals in sexually deceptive orchids of the *Ophrys fusca* group. Pl. Syst. Evol. 254:105-120

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Stökl J., Schlüter P. M., Stuessy T. F., Paulus H. F., Assum G, Ayasse M. (in prep.) Genetic variation, hybridization, and introgression in co-flowering *Ophrys* species on Majorca

Gögler J., Stökl J., Sramkova A., Twele R., Wittko F., Ayasse M. (2007) The role of pollinator attracting scent in the sexually deceptive orchids *Ophrys chestermanii, O. normanii* and *O. tenthredinifera*. (submitted)

Conference contributions
Johannes Stökl, Philipp M. Schlüter, Tod F. Stuessy, Hannes F. Paulus, Manfred Ayasse (2007) Consequences of hybridization on the pollinator attracting odour, genetic variation, and radiation of three *Ophrys* species on Majorca. 23nd Annual Meeting of the International Society of Chemical Ecology, Jena, Germany

Johannes Stökl and Manfred Ayasse (2007) Bestäuberanlockung und Artisolation bei Sexualtäuschorchideen der Gattung *Ophrys* auf Sardinien. Entomologentagung 2007, Innsbruck, Austria

Johannes Stökl, Philipp Schlüter, Tod Stuessy, Manfred Ayasse (2006) Scent variation, hybridization and speciation in sexually deceptive orchids of the genus *Ophrys*. 22nd Annual Meeting of the International Society of Chemical Ecology, Barcelona, Spain

Johannes Stökl and Manfred Ayasse (2006) Variation des Bestäuber anlockenden Blütendufts, Hybridisierung und Artbildung bei Sexualtäuschorchideen der Gattung *Ophrys*.
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Johannes Stökl and Manfred Ayasse (2005) Scent variation and its role in hybridisation and speciation of sexually deceptive orchids of the genus *Ophrys*, 17th International Botanical Congress, Vienna, Austria

Johannes Stökl and Manfred Ayasse (2004) Comparison of pollinator attracting scent in sexually deceptive orchids of the *Ophrys fusca*-group. 12th International Symposium on Insect-Plant Relationships, Berlin, Germany

Johannes Stökl and Manfred Ayasse (2004) Inter- und intraspezifische Variation der bestäuberanlockenden Duftstoffe bei Sexualtäuschorchideen der *Ophrys fusca* Gruppe.
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Johannes Stökl and Manfred Ayasse (2003) Pollinator attracting odour signals in sexually deceptive orchids of the *Ophrys fusca* group. 96. Jahresversammlung der Deutschen Zoologischen Gesellschaft, Berlin, Germany

Awards

Student Travel Award, 23nd Annual Meeting of the International Society of Chemical Ecology, Jena, Germany, 2007

2nd place "Best Student Presentation", 23nd Annual Meeting of the International Society of Chemical Ecology, Jena, Germany, 2007

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Field trips and research stays

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Visiting researcher in Vienna, Sep. 2004 in the lab of Prof. Tod Stuessy
Visiting researcher in Zurich, Feb. 2004 in the lab of Dr. Florian Schiestl

Foreign languages
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Memberships
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Eidesstattliche Erklärung

Hiermit erkläre ich, die vorliegende Dissertationsarbeit selbständig angefertigt und keine anderen als die in der Arbeit aufgeführten Hilfsmittel verwendet zu haben. Wörtlich oder inhaltlich übernommene Stellen wurden als solche gekennzeichnet.

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