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Pollinator attracting odour signals in sexually deceptive orchids of the Ophrys fusca group

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Abstract. We investigated patterns of volatiles of several allopatric and sympatric species of the *Ophrys fusca* group and one species of the *O. mammosa/sphegodes* group pollinated by either *Andrena nigroaenea* or *A. flavipes*, using electrophysiology (gas chromatography coupled with electroantennography; GC-EAD) and chemical analyses. We found 52 GC-EAD active compounds, mainly saturated and unsaturated hydrocarbons with chain lengths of 21 to 31, aldehydes, an ester, and an acid. Based on the relative proportions of all GC-EAD active compounds, the investigated species were compared using various statistical methods (ANOVA, principle component analyses, discriminant function analyses and cluster analyses). Our results show that *Ophrys* species with the same pollinator – independent of their phylogenetic relationship – use the same volatiles for pollinator attraction. Differences between the species mainly involve different quantitative patterns of volatiles. Our results are in congruence with previous studies that showed different odour bouquets to be responsible for the specific attraction of different pollinators and that alkanes and alkenes are most important for pollinator attraction.

Key words: Pollination by sexual deception, pollinator attraction, floral odour, GC-EAD, Orchidaceae, *Andrena*, *Ophrys*.

Introduction

Sexual deception of male bees is one of the most remarkable mechanisms of pollination and is exclusive to the Orchidaceae (Dafni 1984, Ackermann 1986, Nilson 1992). It has been documented in Australia (Peakall 1990), South America (van der Pijl and Dodson 1966, Singer 2002), South Africa (Steiner et al. 1994) and Europe (Kullenberg 1961, Kullenberg and Bergström 1976, Paulus and Gack 1990a). *Ophrys* orchids grow mainly around the Mediterranean (Europe, Northern Africa, Middle East), but are also common in central and northern Europe (Delforge 2001). They are pollinated mostly by bees (Andrenidae, Anthophoridae, Colletidae, Megachilidae, and Apidae) as well as by predatory and parasitic wasps (Sphecidae and Scoliidae) and occasionally by beetles (Scarabaeidae, Kullenberg 1961, 1973; Borg-Karlson 1990; Paulus and Gack 1990a).

Ophrys flowers imitate the pollinators' females in shape, colour and, most importantly, in scent; males of the pollinating bees are lured to the flowers by olfactory signals

and optical cues. Once the male lands on a flower, close-range semiochemicals elicit sexual behaviour similar to that released by the female's sex-pheromone, and the males try to copulate with the flower labella (Kullenberg 1961). This phenomenon was first described by Pouyanne (1917) and is a classical example of odour communication, which is reasonably well understood today (Bergström 1978; Schiestl et al. 1999, 2000; Ayasse et al. 2000, 2003). Pollination in *Ophrys* is usually highly specific, with only a single pollinating species visiting each *Ophrys* species. Only in rare cases is pollination performed by a few closely related species (Kullenberg 1961, 1973; Paulus and Gack 1986, 1990a, 1994). The highly specific *Ophrys*–pollinator relationship represents the main mechanism of reproductive isolation between the often intercrossable *Ophrys* species (Bergström 1978, Ehrendorfer 1980, Paulus and Gack 1990a).

The complex species-specific bouquets of semiochemicals released by *Ophrys* flowers often consist of more than 100 different volatile compounds (Borg-Karlson et al. 1985, 1987; Ayasse et al. 2000). Several investigations have shown that only certain subsets of all the compounds produced by *Ophrys* flowers are important to release pseudocopulatory behaviour in the males (Kullenberg and Bergström 1976; Tengö 1979; Borg-Karlson 1990; Schiestl et al. 1999, 2000; Ayasse et al. 2003). Schiestl et al. (1999) found that only 14 compounds of the *Ophrys sphegodes* bouquet, namely alkanes and alkenes, are responsible for releasing copulation attempts in *Andrena nigroaenea* males. These 14 compounds were found in similar proportions both in extracts of the *O. sphegodes* flower labella and in cuticle surface extracts of virgin pollinator females. In the orchids *O. fusca* and *O. bilunulata* Schiestl and Ayasse (2002) showed that only slight differences in the relative proportions of alkanes and alkenes induce species-specific attraction of pollinators, while almost all the compounds are produced by both species.

In this study, we investigated five species of the *O. fusca*-complex (Delforge 2001, Foelsche and Foelsche 2001). The name *O. fusca* Link s.l. was originally used for only one species in the whole Mediterranean basin, but more detailed investigations showed that there are at least 29 species, some of them difficult to distinguish by morphological traits (Delforge 2001, Paulus 2001). In these species, genetic isolation is mediated by different pollinator species. Plants flower in spring and usually bear two to six flowers.

Many species of the *O. fusca*-group are pollinated by males of *Andrena* bees. *A. nigroaenea* and *A. flavipes* are two of the most common species of this genus. Both species occur sympatrically, and females nest in sandy soil. *A. flavipes* is bivoltine with one generation in spring and a second generation in late summer (Schmid-Egger and Scheuchl 1997). In their search for females, males of both species patrol along typical landmarks and around the nests of the females (Westrich 1989).

Three of the investigated species, *O. bilunulata* Risso, *O. israelitica* Baumann & Künkele and *O. africana* G. & W. Foelsche, are pollinated by *A. flavipes* Panzer (Paulus 2001). *O. bilunulata* was described to occur in the whole Mediterranean basin, but in the present literature, it is split into several species with distinct distributions (Delforge 2001): *O. africana*, O. leucadica Renz, *O. funerea* Viviani and *O. obaesa* Lojacono; the name *O. bilunulata* is currently only used for plants that occur in the northwestern Mediterranean basin (Paulus 1988, 2001). Furthermore, according to morphometric analyses of the flowers, *O. africana* is supposed to be identical with *O. gazella* (Tunesia) and *O. caesiella* (Malta) (Foelsche and Foelsche 2004). Consequently, all of them should be placed as geographical subspecies of *O. bilunulata*. Unfortunately, the whole *Ophrys*-systematics and, above all, nomenclature differ greatly according to different authors (Renz 1929; Baumann and Künkele 1982, 1986, 1988; Delforge 2001; Paulus 2001). Two investigated species, *O. fusca* and *O. sitiaca* Paulus, C. & A. Alibertis are pollinated by *A. nigroaenea* Kirby (Paulus 2001). We also investigated *O. herae*

Hirth & Spaeth, which is part of the *O. mammosa/sphegodes*-group in Greece (Delforge 2001). *O. herae* is pollinated by *A. nigroaenea*, but it places the pollinia on the head of the pollinator, while all species of the *O. fusca*-group place their pollinia on the tip of the bees' abdomen. Therefore it can co-occur sympatrically with *O. fusca* and *O. sitiaca* without risk of hybridisation.

Except for two species (Schiestl and Ayasse 2002), it is unknown whether allopatrically occurring *Ophrys* species that have the same pollinator also use the same compounds for pollinator attraction. Using electrophysiological methods in combination with gas-chromatography (GC-EAD), as well as gas chromatography in combination with mass spectrometry (GC-MS), we compared the GC-EAD active volatile signals of allopatrically and sympatrically occurring *Ophrys* species visited by identical pollinator species.

The objectives of this study were to determine:

1) whether *Ophrys* species with the same pollinator use the same volatile compounds in quantitatively identical blends to attract their pollinators; 2) how the blends of volatiles differ among *Ophrys* species that have different pollinators; 3) whether the chemical composition of volatiles can provide information on the phylogenetic relationship between the species in the *O. fusca* group.

Materials and methods

Sample collection. *Ophrys* plants were collected at four different sites (Table 1). Single floral labella were extracted in either 500 or 1000 µl pentane (depending on the size of the labellum) for 48 h, after which the labella were removed and the extract samples stored in the freezer at −20°C. Before chemical analyses, the samples were concentrated to 70 µl, and 1 µg octadecane (C18) was added to each sample as internal standard.

Males of *A. nigroaenea* and *A. flavipes* were collected at the Institute of Zoology in Vienna and near Oberweiden in Lower Austria.

Gas chromatography and Electrophysiology (GC-EAD). Gas chromatography with electroantennographic detection (GC-EAD) was used to detect which of the volatile chemicals of the *Ophrys* flowers are perceived by the male bee's antennae. For each EAD the tip of an excised antenna was cut off and the antenna mounted between two glass-electrodes filled with insect ringer. The electrode at the antenna's base was grounded via an Ag-AgCl wire and the electrode at the tip of the antenna was connected via an amplifier to a signal interface board (Syntech, Hilversum, Netherlands) of a PC. One µl of the extract was injected splitless into a gas chromatograph HP6890 (Hewlett-Packard, Palo Alto, CA) at 50°C. After 1 min the split valve was opened and the temperature increased by 10°C min^{-1} up to 310°C. The GC was equipped with a DB5 capillary column (30m × 0.32mm i.d. J&W) and a FID, and helium served as the carrier gas. The effluent was split (variable outlet splitter (SGE, Darmstadt, Germany); split ratio FID:EAD = 1:3) and the outlet for the EAD was placed in a cleaned and humidified airflow that was directed over the male bee's antenna. The outlet was heated (310°C) to avoid condensation of the effluent in the cooler airflow. EAD and FID signals were recorded simultaneously on a PC running a GC-EAD program (Syntech, Hilversum, Netherlands).

GC-EAD runs were obtained with *O. sitiaca* and *O. herae* using antennae of *A. nigroaenea*, and

Table 1. Investigated *Ophrys* species with their corresponding pollinators, and sample collection information

Species	Pollinator	Sample Site	Date	N
O. fusca	*A. nigroaenea*	Majorca	Feb. 1998	9
O. sitiaca	*A. nigroaenea*	Crete	Feb. 2001	15
O. bilunulata	*A. flavipes*	Majorca	Mar. 1998	13
O. africana	*A. flavipes*	Tunisia	Feb. 1996	11
O. israelitica	*A. flavipes*	Israel	Mar. 2001	13
O. herae	*A. nigroaenea*	Crete	Feb. 2001	13

with *O. israelitica* using antennae of *A. flavipes*. For each *Ophrys* species, approximately 10 runs were performed. EAD peaks were termed "active" when they could be recorded in at least 50 percent of the runs at exactly the same retention time. GC-EAD active compounds were identified by performing GC-EAD runs using reference substances based on GC-MS analysis (with samples of *O. sitiaca* and *O. herae*). Absolute amounts of the biologically active compounds were calculated using HP Chem-Station software (Hewlett-Packard, Palo Alto, CA). The electrophysiologically active compounds of *O. fusca* and *O. bilunulata* have already been described by Schiestl and Ayasse (2002).

Gas chromatography (GC). The *Ophrys* labella extracts were analysed using a HP5890 Series II gas-chromatograph (Hewlett-Packard, Palo Alto, CA), equipped with a DB5 capillary column (30 m * 0.32 mm i.d.) and helium as the carrier gas. One µl of the sample was injected splitless at 120°C. After 1 min the split valve was opened and temperature increased by 4°C min^{-1} up to 290°C.

Structure elucidation of individual compounds was based on GC-MS analysis (VG70/250 SE instrument, Vacuum Generators, Manchester, England, linked to a HP 5890 gas chromatograph; condition as mentioned above). Mass spectra were compared with those reported in the literature (Mc Lafferty and Stauffer 1989) and gas chromatographic retention times (coinjection) with those of authentic reference samples. Double bond positions in unsaturated compounds were assigned according to Buser et al. (1983) and Dunkelblum et al. (1985).

Statistics. Statistical analyses were conducted according to Ayasse et al. (2000). To determine possible differences and similarities between species or groups of species visited by different pollinators, we used relative proportions of the GC-EAD active compounds. Statistical analyses were performed with arc-sinus transformed data and four datasets, namely hydrocarbons, alkanes, alkenes/alkadienes and non-hydrocarbons. To reduce the number of variables, a principle component analysis (PCA; varimax rotation) was performed. Resulting principal components with an eigenvalue above one were used for discriminant function analysis (DFA). Two DFAs were performed for each data set, one with the *Ophrys* samples grouped by species, the other with the samples grouped by pollinator. 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. Hierarchical cluster analyses (Wards Method) were calculated with the discriminant scores. Cluster analyses were performed with group means of discriminant functions and for separate samples. Means of relative amounts of alkanes and alkenes were tested for significant differences with an ANOVA (Analysis of Variance, Tamhane post hoc test). All statistics were performed with SPSS 7.5 (SPSS 1997).

Results

Biologically active compounds. We found 46 peaks, comprising 52 chemical compounds, to be active in the simultaneous recordings of FID and EAD signals. Some of the samples were contaminated with silicones. These contaminations coeluted with some of the GC-EAD active peaks (X1, X2, X3, X5, X9, X13, (*Z*)-9-nonacosene) that were, therefore, excluded from further analyses and not listed in Table 2. In *O. sitiaca* we registered 25 peaks consisting of 28 active compounds (Table 2, Fig. 1a), in *O. herae* 31 peaks comprising 37 compounds (Table 2, Fig. 1b). The highest number of active compounds was found in *O. israelitica*, with 39 compounds in 36 peaks (Table 2, Fig. 1c). Although most of the electrophysiologically active compounds were present in all investigated *Ophrys* species, they did not always trigger EAD responses. GC-EAD runs with reference compounds showed that this was due to the low concentration of these compounds in the bouquets of certain investigated species.

The biologically active compounds identified were mainly hydrocarbons, namely heneicosane to heptacosane (C21–C27), alkenes C25 to C31 showing the double bond at positions 7, 9, 11 to 14, and alkadienes C27, C29, and C31 (unknown double-bond positions). Among the non-hydrocarbons, aldehydes (chain lengths 9 to 26) were active, as were also nonanoic acid and one wax-type ester, 2-nonyl hexadecanoate (unknown enan-

Table 2. Mean relative amounts (%) of the electrophysiologically active compounds in the labella extracts of the investigated *Ophrys* species. Percentage was calculated separately for alkanes, alkenes/ alkadiens and non-hydrocarbons

No.	Compound	*Ophrys fusca*	*Ophrys sitiaca*	*Ophrys herae*	*Ophrys israelitica*	*Ophrys bilunulata*	*Ophrys africana*	Sign. difference between species (ANOVA, post-hoc Tamhane $p < 0.05$)[d]
Alkanes		mean ± SE	mean ± SE	mean ± SE	mean ± SE	mean ± SE	mean ± SE	
1	Heneicosane	0.67 ± 0.4	0.91 ± 0.4*	1.54 ± 0.3*	3.13 ± 1.9*	3.77 ± 2.5	1.59 ± 0.7	FA;FH;FI;FB;SH;SI;SB
2	Docosane	0.96 ± 0.3	0.89 ± 0.5	2.42 ± 0.6*	1.37 ± 0.4*	0.97 ± 0.6	0.56 ± 0.3	FH;AH;AI;SH;HI;HB
3	Tricosane	45.41 ± 6.6	22.39 ± 8.4*	37.14 ± 7.4*	27.62 ± 7.5*	50.28 ± 4.8	37.47 ± 7.8	FS;FI;AS;AB;SH;SB;HI;HB;IB
4	Tetracosane	5.45 ± 1.8	4.23 ± 1.2*	5.82 ± 0.9*	3.68 ± 0.7*	3.44 ± 0.5	3.23 ± 0.5	AH;SH;HI;HB
5	Pentacosane	19.75 ± 3.1	36.49 ± 4.7*	31.57 ± 3.7*	27.60 ± 3.8*	22.18 ± 4.3	26.07 ± 3.4	FA;FS;FH;FI;AS;AH;SI;SB;HB;IB
6	Hexacosane	1.38 ± 0.6	2.13 ± 0.5*	3.31 ± 0.8*	3.77 ± 0.8*	1.53 ± 0.4	2.59 ± 0.5	FA;FH;FI;AI;AB;SH;SI;SB;HB;IB
7	Heptacosane	26.38 ± 6.2	32.95 ± 8.4*	18.21 ± 3.9*	32.82 ± 6.6*	17.83 ± 3.7	28.48 ± 5.7	FB;AH;AB;SH;SB;HI;IB
Alkenes/ alkadienes								
8	(*Z*)−12/(*Z*)−11-Pentacosene[a]	8.50 ± 8.2	7.62 ± 6.8*	4.48 ± 3.2*	0.67 ± 0.7*	1.99 ± 1.0	1.28 ± 1.1	AS;SI;HI;IB
9	Heptacosadiene[b]	4.90 ± 4.8	8.67 ± 11.5*	6.15 ± 5.7*	4.64 ± 5.4*	2.16 ± 1.2	0.68 ± 0.2	AB
10	(*Z*)−13/(*Z*)−12/(*Z*)-11-Heptacosene[a]	23.18 ± 5.6	20.92 ± 6.2*	20.17 ± 5.6*	12.22 ± 3.6*	15.04 ± 8.1	17.47 ± 4.9	FI;SI;HI
11	(*Z*)−9-Heptacosene	13.71 ± 3.5	18.56 ± 5.6*	21.88 ± 5.6*	19.66 ± 6.6*	20.94 ± 6.3	23.32 ± 4.3	FA;FH;FB
12	(*Z*)−7-Heptacosene	4.30 ± 3.8	0.00 ± 0.0	0.51 ± 0.3	4.46 ± 2.9*	10.47 ± 6.2	16.22 ± 5.7	FA;AS;AH;AI;SH;SI;SB;HI;HB
13	Nonacosadiene[b]	12.45 ± 2.4	13.53 ± 2.9*	16.10 ± 6.1*	8.45 ± 1.9	6.78 ± 4.1	6.60 ± 4.4	FA;FI;FB;AS;AH;SI;SB;HI;HB
14	Nonacosadiene[b]	12.83 ± 5.0	16.99 ± 7.4*	14.48 ± 4.4*	22.78 ± 5.2*	16.79 ± 5.9	14.37 ± 3.1	FI;AI;HI
15	(*Z*)−14/(*Z*)−13-/(*Z*)-12/(*Z*)-11-Nonacosene[a]	4.84 ± 6.1	0.00 ± 0.0	9.01 ± 7.1*	8.70 ± 9.9	13.98 ± 11.5	14.86 ± 5.6	FA;AS;SH;SB
16	Hentriacontadiene[b]	15.29 ± 6.8	13.71 ± 4.6	7.22 ± 3.5	18.43 ± 6.2*	11.85 ± 6.5	5.19 ± 2.2	FA;AS;AI;AB;SH;HI
Non-hydrocarbons and unknown compounds								
17	Nonanal	5.16 ± 1.5	6.02 ± 3.5*	9.37 ± 6.5*	6.49 ± 2.5*	3.28 ± 1.5	13.33 ± 1.9	FA;AS;AI;AB;IB
18	Decanal	3.58 ± 1.3	3.76 ± 1.2	1.40 ± 0.5*	2.14 ± 0.7*	1.30 ± 1.0	0.72 ± 0.3	FA;FH;FB;AS;AH;AI;SH;SI;SB

Table 2. (Continued)

No.	Compound	*Ophrys fusca*	*Ophrys sitiaca*	*Ophrys herae*	*Ophrys israelitica*	*Ophrys bilunulata*	*Ophrys africana*	Sign. difference between species (ANOVA, post-hoc Tamhane $p < 0.05$)[d]
Alkanes		mean ± SE	mean ± SE	mean ± SE	mean ± SE	mean ± SE	mean ± SE	
19	Nonanoic acid	2.57 ± 2.1	4.81 ± 3.7*	5.98 ± 4.1*	5.54 ± 2.7*	2.10 ± 4.1	4.05 ± 1.9	—
20	Undecanal	6.09 ± 3.1	3.71 ± 3.7*	2.40 ± 2.3*	1.15 ± 1.0*	3.61 ± 4.0	1.62 ± 0.7	FA;FI
21	Dodecanal	4.31 ± 2.6	3.66 ± 1.5*	1.81 ± 0.6*	1.83 ± 0.8*	0.99 ± 0.7	0.40 ± 0.1	FA;AS;AH;AI;SH;SI;SB
22	Tridecanal	2.84 ± 1.3	1.24 ± 0.5	2.50 ± 1.2*	1.14 ± 0.5*	1.01 ± 0.7	1.11 ± 0.3	FB;AH;SH;HI;HB
23	Hexadecanal	2.33 ± 0.8	2.16 ± 0.8*	3.26 ± 0.9*	1.29 ± 0.5*	1.41 ± 0.8	1.34 ± 0.4	FI;AS;AH;SH;SI;HI;HB
24	Octadecanal	5.80 ± 1.7	6.43 ± 2.7*	8.70 ± 2.7*	5.67 ± 2.5*	4.93 ± 2.8	4.41 ± 1.2	AH;HB
25	Nonadecanal	9.97 ± 5.6	9.64 ± 3.6*	1.22 ± 0.5*	4.19 ± 2.6*	2.42 ± 2.3	0.64 ± 0.1	FA;FH;FB;AS;AH;AI;SH;SI;SB;HI
26	X4	11.79 ± 14.4	14.67 ± 13.9*	11.16 ± 11.8*	14.18 ± 13.9*	14.53 ± 14.2	5.89 ± 3.0	—
27	Eicosanal	5.84 ± 2.3	9.58 ± 4.0*	18.70 ± 4.5*	15.63 ± 5.5*	14.38 ± 6.3	25.23 ± 3.4	FA;FH;FI;FB;AS;AH;AI;AB;SH;SI
28	X6	0.43 ± 0.3	0.25 ± 0.3*	0.01 ± 0.0*	0.16 ± 0.2*	0.08 ± 0.2	0.10 ± 0.1	—
29	Heneicosanal	4.97 ± 2.6	3.48 ± 1.4	2.18 ± 1.4	2.21 ± 0.9*	1.04 ± 0.6	0.57 ± 0.1	FA;FB;AS;AH;AI;IB
30	X9	1.64 ± 1.3	0.48 ± 0.4	1.83 ± 1.5*	0.93 ± 0.9*	1.63 ± 0.7	0.75 ± 0.5	AB;SB
31	X10	2.41 ± 4.6	0.00 ± 0.0	0.95 ± 0.3	0.20 ± 0.2*	0.03 ± 0.1	0.23 ± 0.1	AS;AH;AB;SH;HI;HB
32	Docosanal	3.61 ± 2.0	8.74 ± 4.3	6.59 ± 2.3	10.82 ± 4.3*	12.87 ± 3.3	16.59 ± 4.7	FA;FS;FI;FB;AS;AH;HB
33	2-Nonyl hexadecanoate[c]	2.88 ± 2.6	1.23 ± 1.5*	4.47 ± 4.2*	1.46 ± 1.1*	0.64 ± 0.3	0.63 ± 0.4	—
34	X12	0.31 ± 0.3	0.69 ± 0.7	0.48 ± 0.5	0.64 ± 0.6*	0.22 ± 0.2	0.06 ± 0.1	AS
35	Tetracosanal	6.56 ± 2.9	7.18 ± 5.2*	2.30 ± 0.7*	5.57 ± 1.2*	9.41 ± 3.2	4.76 ± 1.0	FH;AH;AB;SH;HI;HB;IB
36	X14	0.39 ± 0.4	0.20 ± 0.3	0.09 ± 0.3	1.54 ± 1.0*	0.42 ± 1.3	0.17 ± 0.2	FI;AI;SI;HI
37	X16	9.10 ± 3.8	8.83 ± 5.0	11.22 ± 11.9*	12.69 ± 7.4	15.47 ± 8.6	7.80 ± 3.3	—
38	X17	0.33 ± 0.4	0.43 ± 0.3*	1.79 ± 1.4*	0.50 ± 0.2*	0.83 ± 0.9	0.66 ± 0.3	FH
39	Hexacosanal	7.09 ± 4.2	2.84 ± 2.2	1.60 ± 0.6	4.04 ± 1.4*	7.41 ± 4.1	8.94 ± 3.4	AS;AH;AI;SB;HI;HB

EAD response obtained in this study

a) Compounds that could not be separated with the GC-parameters used

b) Double-bond positions unknown

c) Enantiomeric composition unknown

d) Pairs of letters indicate significant differences between those species F = ***O. fusca***, S = ***O. sitiaca***, H = ***O. herae***, I = ***O. israelitica***, B = ***O. bilunulata***, A = ***O. africana***

tiomeric composition, Table 2). Fourteen electrophysiologically active compounds could not yet be identified. In all investigated *Ophrys* species alkanes and alkenes released the strongest reactions in male antenna (Fig. 1a–c).

The relative proportions of active alkanes, alkenes and non-hydrocarbons of all investigated species are shown in Table 2. Alkanes were dominated by the uneven numbered tricosane, pentacosane and heptacosane. Alkenes present in the highest proportions in the samples were (*Z*)-heptacosenes (double bond positions 13, 12, 11, 9) and nonacosadiene.

Most of the non-active compounds belong to the same chemical classes as the active compounds and differences were mainly in the chain lengths and the double-bond positions (Ibarra 2002). Further non-active compounds

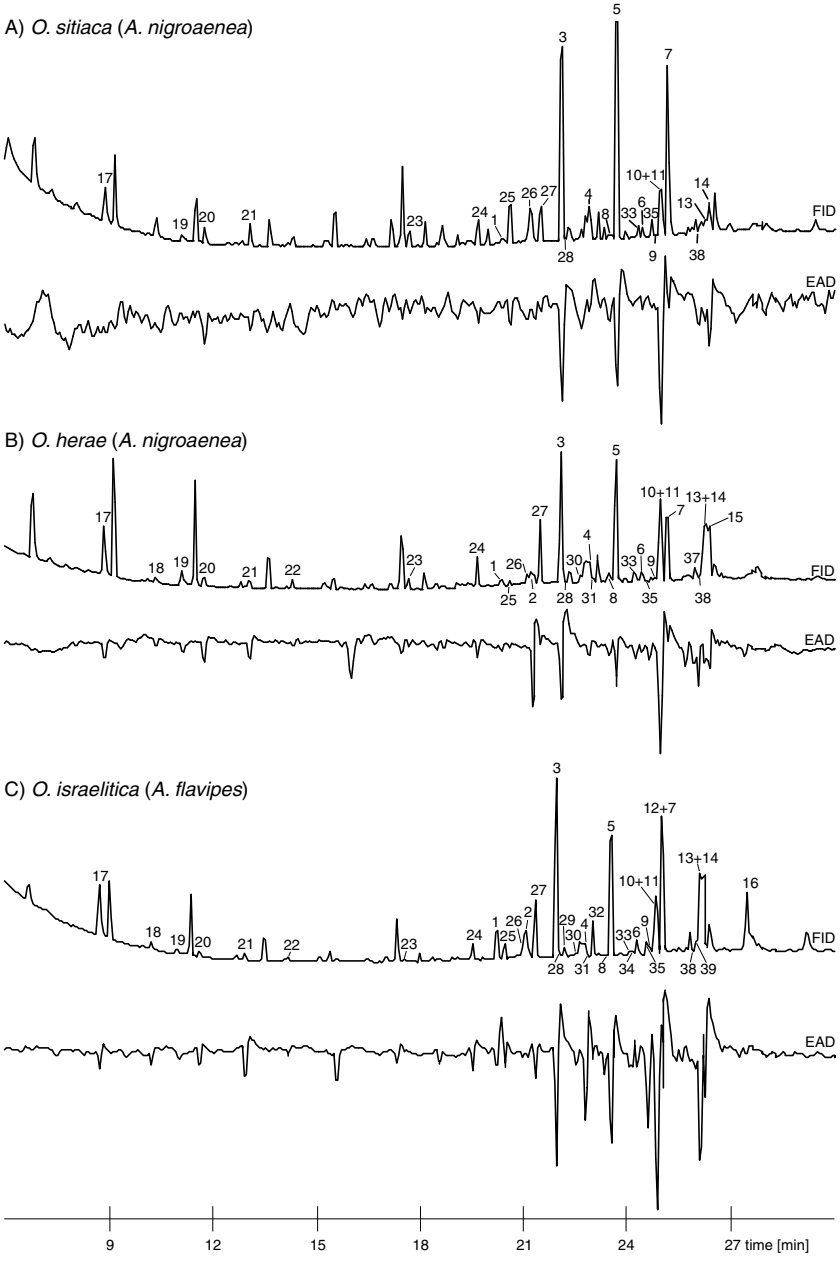


Fig. 1. Simultaneous recordings of FID and EAD signals of **a**) *O. sitiaca* **b**) *O. herae* and **c**) *O. israelitica* labella extracts using male antennae of the particular pollinating bee species. The highest reactions in male antennal receptors were triggered by alkanes and alkenes. Electrophysiologically active peaks are numbered according to Table 2

have been identified as fatty acids and esters of fatty acids.

Interspecific variation in the odour bouquets. Three principal components (PCs) with an eigenvalue above one explained 84.4% of the total matrix variance in alkanes. The first PC was mainly associated with C23, C25, C26 and C27, the second PC with C22 and C24, and the third PC with C21.

In the PCA performed with alkenes/alkadienes, three PCs with an eigenvalue above one explained 71.1% of variance. The first PC was associated with alkenes and alkadienes of higher carbon chain lengths (C27 and C29), the second and third PC were mainly linked with alkenes/alkadienes C25 and C27 (different double-bond positions than in the first PC).

The PCA based on all hydrocarbons generated 16 PCs, five of them with an eigenvalue above one, explaining 81.2% of variance. In the first PC, three alkanes (C23, C24, and C25) and four alkenes/alkadienes C27 and C31 had the major factor loadings. The second PC was mainly associated with alkenes C27 and C29, the third PC was associated with C27-diene, docosane and heptacosadiene. In PC four the short-chain alkane C21 and alkenes/alkadienes C25, C27, and C29 had the main factor loadings. The fifth PC was linked with tricosane and hexacosane.

In the non-hydrocarbons, six PCs with an eigenvalue above one explained 74.0% of the total variance; 23 PCs were needed to explain all the variance. The high number of PCs compared to the other compound classes indicates a higher variance in non-hydrocarbons. Most of the PCs were mainly associated with aldehydes.

Species-dependent and pollinator-dependent bouquets of volatiles. A comparison with a discriminant function analysis (DFA), based on the factor scores of the PCs for alkanes, showed significant differences between species. Of all cases 94.6% (79.9% at cross-validation, c.v.) were categorized to the correct species by three significant discriminant functions (DF 1: $x^2 = 242.1$, $df = 15$, $p < 0.001$, DF2: $x^2 = 134.8$, $df = 8$, $p < 0.001$, DF3: $x^2 = 50.0$, $df = 3$, $p < 0.001$). Standardized canonical discriminant function coefficients and factor scores showed that PC one and two, representing all alkanes except heneicosane, were relevant for separation by species. A further DFA ($x^2 = 82.6$, $df = 3$, $p < 0.001$) for separation by pollinator was significant as well, and the differences were mainly in the concentrations of the short-chain alkanes C21, C22, and C24. 93.2% (93.2% at c.v.) of the flowers were classified correctly according to the pollinating bee.

In a DFA based on alkenes/alkadienes, 62.2% (52.7% at c.v.) of the cases were classified to the correct species (DF1: $x^2 = 148.4$, $df = 15$, $p < 0.001$, DF2: $x^2 = 43.1$, $df = 8$, $p < 0.001$, DF3: $x^2 = 6.2$, $df = 3$, $p = 0.102$). In a further DFA ($x^2 = 85.2$, $df = 3$, $p < 0.001$), 90.5% (89.2% at c.v.) of the cases were classified according to the pollinating bee. Standardized canonical discriminant function coefficients and factor scores showed that the differences between species visited by different pollinators are based on the relative amounts of alkenes C27 and C29 and alkadienes C29 and C31. Therefore, patterns of alkenes/alkadienes are most important for separating *Ophrys* species pollinated by different pollinators.

When all hydrocarbons were included to perform a DFA by species (DF1: $x^2 = 319.9$, $df = 25$, $p < 0.001$, DF2: $x^2 = 182.5$, $df = 16$, $p < 0.001$, DF3: $x^2 = 99.6$, $df = 9$, $p < 0.001$, DF4: $x^2 = 26.2$, $df = 4$, $p < 0.001$, DF5: $x^2 = 0.3$, $df = 1$, $p = 0.557$), 94.6% (90.5% at c.v.) of all cases were correctly classified. The first discriminant function explaining 57.8% of the variance had the highest standardized canonical discriminant function coefficients on PCs two and four of the PCA. Therefore, all alkenes and four alkanes were most important for separating the *Ophrys* species. The second function explained an additional 21.0% and had the highest coefficient on PC three of the PCA. Of all cases 100% (100% at c.v.) were correctly classified to their pollinator. All variance was explained by one discriminant function ($x^2 = 134.0$, $df = 5$, $p < 0.001$), which

had the highest coefficients on PCs two and four of the PCA (same as in the DFA by species).

A DFA based on the non-hydrocarbons could classify 89.2% (85.1% at c.v.) of all cases to the correct *Ophrys* species (DF1: $x^2 = 324.8$, $df = 30$, $p < 0.001$, DF2: $x^2 = 179.5$, $df = 20$, $p < 0.001$, DF3: $x^2 = 88.2$, $df = 12$, $p < 0.001$, DF4: $x^2 = 29.5$, $df = 6$, $p < 0.001$, DF5: $x^2 = 11.4$, $df = 2$, $p < 0.01$). Standardized canonical discriminant function coefficients and factor scores of the first two discriminant functions, explaining 84.9% of variance, showed nearly all found aldehydes to be important for separating the *Ophrys* species. The separation by pollinator was correct at

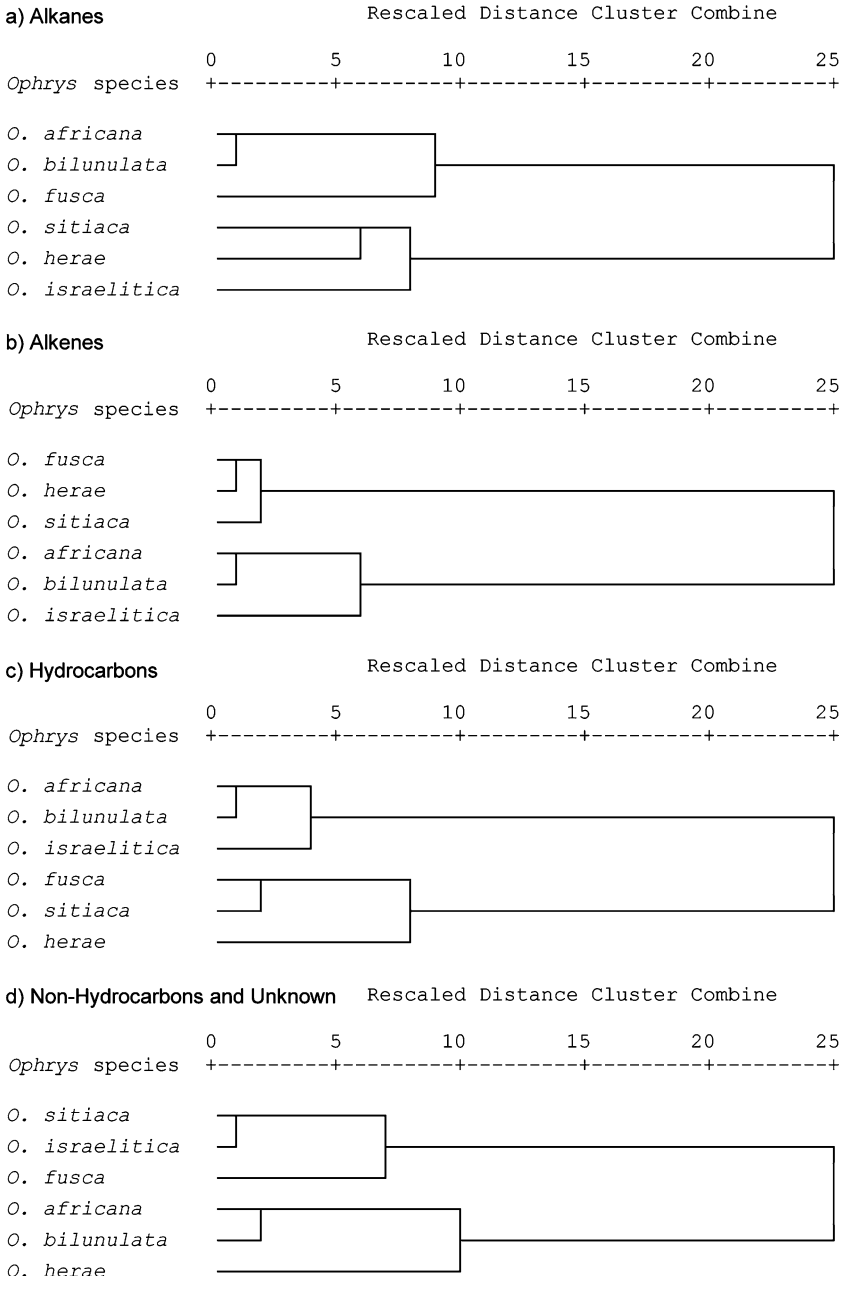
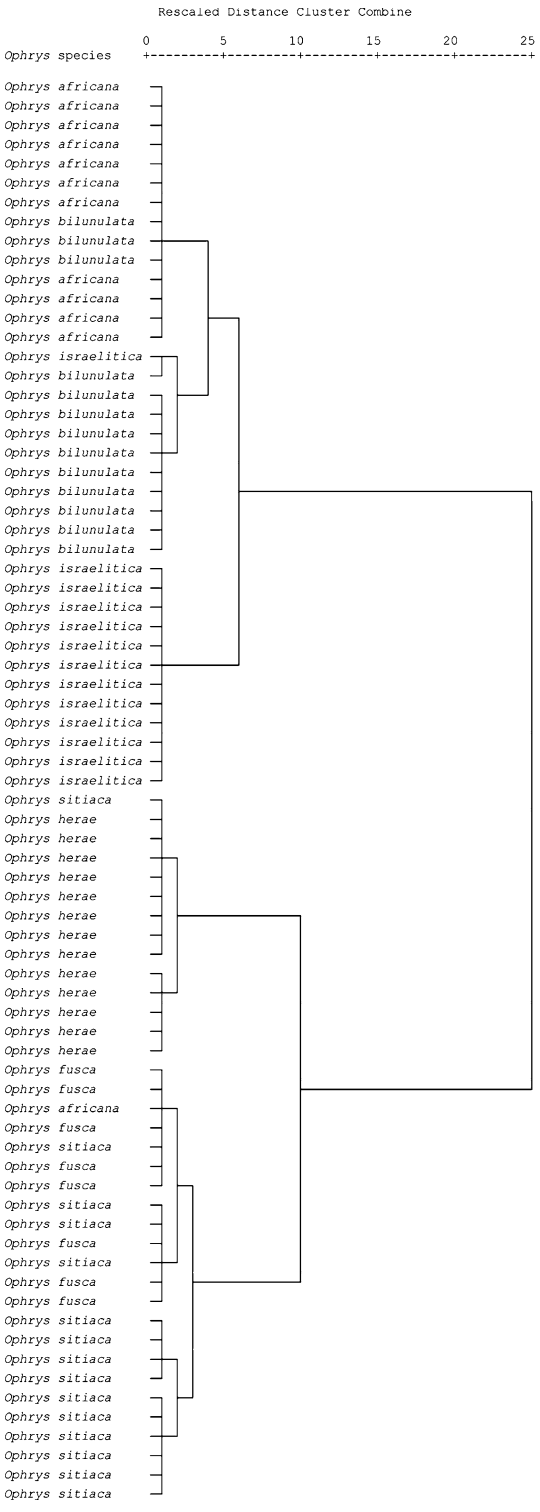


Fig. 2. Similarity in floral scent among the investigated *Ophrys* species based on the datasets of different chemical classes. We used discriminant function scores of group means to perform cluster analyses using Ward's method. In the underlying DFA samples were grouped by *Ophrys* species. Dendrograms for alkenes (**b**) and hydrocarbons (**c**) show a clear separation of species with the same pollinating bee species, whereas the ones for alkanes (**a**) and non-hydrocarbons (**d**) show neither a separation by pollinator nor a conformance with the current systematics (*O. herae*, part of the *O. mammosa*/*sphegodes* group, is not separated from the species of the *O. fusca* group)



90.5% (89.2% at c.v.). The function ($x^2 = 76.3$, $df = 6$, $p < 0.001$) was correlated mainly with the first PC, comprising eight aldehydes.

Fig. 3. Similarity in the patterns of hydrocarbons in the floral scent among the investigated *Ophrys* specimen. We used discriminant function scores to perform cluster analyses using Ward's method. In the underlying DFA samples were grouped by *Ophrys* species. Individuals of species with the same pollinating bee species are grouped together in the two main clusters. However, two individuals of *O. fusca,* although pollinated by *A. nigroaenea*, are placed in the cluster of the specimen pollinated by *A. flavipes*. Within each cluster, individuals of a single species are not always completely separated into their own cluster

In a cluster analysis performed with the relative proportions of alkanes, based on the means of discriminant functions, *Ophrys* species with the same pollinator were clustered neither according to their pollinator nor to the phylogenetic relationship of the species (Fig. 2a). In a cluster analysis performed with relative proportions of alkenes/alkadienes, all *Ophrys* species pollinated by the same bee species were put in the same clusters (Fig. 2b). Within the cluster of species pollinated by *A. flavipes*, *O. bilunulata* and *O. africana,* were placed in a common subcluster. The cluster of all species pollinated by *A. nigroaenea* showed a subcluster containing *O. fusca* and *O. herae*.

In a cluster analysis based on all hydrocarbons, species were again grouped according to their pollinators (Fig. 2c). Within the main cluster containing *Ophrys* species pollinated by *A. flavipes*, *O. bilunulata* and *O. africana* were combined in a common cluster. Among all *A. nigroaenea*-pollinated *Ophrys* species, the two species belonging to the *O. fusca* group formed a single cluster. *O. herae*, part of the *O. mammosa*/*sphegodes*-group, was placed separately.

In the dendrogram resulting from a cluster analysis performed with non-hydrocarbons (aldehydes, esters, acids) and calculated with means of discriminant functions for species, neither the species with the same pollinator nor the species of the *O. fusca*-group formed a common cluster (Fig. 2d). Only the closely

related species *O. bilunulata* and *O. africana* formed a subcluster.

With the dataset of all hydrocarbons, a cluster analysis with values of the discriminant functions for single cases resulted in a dendrogram with two main clusters, each containing almost all cases of *Ophrys* species with the same pollinator (Fig. 3).

Only one sample of *O. africana* was placed with the samples of *Ophrys* species pollinated by *A. nigroaenea*. Within the main cluster of *Ophrys* specimen pollinated by *A. flavipes*, all samples but one of *O. israelitica* were put in a single cluster. A further cluster contained all samples of *O. bilunulata* and *O. africana*. Separation of these two species in subclusters was not perfect, with four wrongly placed samples. Within the cluster of *A. nigroaenea*-pollinated *Ophrys* species, the samples of *O. herae* formed their own subcluster with one misplaced sample of *O. sitiaca*. Some of the samples of *O. sitiaca* formed their own subcluster, while some others were put in a common cluster with samples of *O. fusca*.

Discussion

Electrophysiologically active compounds. Some of the compounds that triggered GC-EAD responses in the present study have been shown to be active in earlier investigations too (all alkanes, six alkenes and two aldehydes; Schiestl et al. 2000, Schiestl and Ayasse 2002). In a comparison of electrophysiologically active compounds in the labella extracts of *O. fusca* and *O. bilunulata*, Schiestl and Ayasse (2002) found 16 peaks comprising 19 compounds, while here we registered 46 peaks comprising 52 chemical compounds. One reason for the higher number of compounds might be the higher number of *Ophrys* species included in our analyses. An alternative explanation, however, could be the improved EAD technique we used. As a consequence of employing a variable outlet splitter and directing a large part of the compounds onto the antennae, we found a higher intensity in the EAD responses. Furthermore, we used make-up gas to direct the volatiles to the antennae. This results in less peak broadening and in a reduction in both residence time and condensation of compounds in the transfer line. This yields sharper and more intense EAD peaks (Schiestl and Marion-Poll 2002).

In comparison to former investigations (Schiestl and Ayasse 2002), we did not find farnesol and farnesyl hexanoate in our samples. Both compounds were found to be produced by pollinated flowers of *O. sphegodes*. Bioassays showed a repellent effect on *Andrena* males, whereby they guide pollinators away from pollinated flowers of an inflorescence (Schiestl and Ayasse 2001). However, the floral scents we investigated were collected from unpollinated flowers, which in *O. sphegodes* produce farnesol and farnesyl hexanoate only in trace amounts (Schiestl and Ayasse 2001). Furthermore, it has not yet been studied whether the species we investigated here also produce farnesol and farnesyl hexanoate after pollination like *O. sphegodes*.

Compounds important for pollinator attraction. In certain species of *Andrena* bees, alkanes and alkenes are most important for eliciting mating behaviour in males (Schiestl et al. 1999, Ayasse et al. 2001) and consequently are used by *Ophrys* flowers to attract *Andrena* males for pollination (Schiestl et al. 1999, 2000). Furthermore, it has been found that different species of *Ophrys* pollinated by different bee species attract their pollinators by different patterns of more or less the same hydrocarbons and not by qualitatively different volatiles (Schiestl and Ayasse 2002). Our results here support these findings in the following ways: 1) Alkanes and alkenes in *Ophrys* species that have the same pollinator occurred in very similar proportions, and the same hydrocarbons triggered receptor potentials in the male antennae. Furthermore, the antennae of both investigated pollinator species, *A. nigroaenea* and *A. flavipes*, showed reactions to most of the odour compounds. Only three alkenes in the samples of *O. israelitica* that were EAD active on the antennae of *A. flavipes* did not release EAD signals in

antennae of *A. nigroaenea*. 2) In the DFA performed with hydrocarbons, there was a highly significant separation of plant specimens grouped by the pollinators and all cases were correctly classified to their pollinating species. 3) In a cluster analysis performed with the GC-EAD active non-hydrocarbons (aldehydes, ester, acid), neither species with the same pollinator nor species of the *O. fusca*-group formed a common cluster (Fig. 2d). Non-hydrocarbons may have a function in influencing flower visitation behaviour in order to increase the reproductive success of a plant, as was shown in previous studies for *O. sphegodes* (Ayasse et al. 2000), which is also pollinated by males of *A. nigroaenea*.

However, one question that remains is which of these hydrocarbons are most important for pollinator-specific attraction? According to our investigations, the patterns of alkenes were almost identical in all species with the same pollinator, and differed in species with different pollinators, indicating their importance for pollinator attraction. This hypothesis is supported by former investigations (Schiestl et al. 2000). In bioassays performed with synthetic mixtures, the alkenes elicited copulation attempts in males of *A. nigroaenea*, whereas alkanes stimulated less intense reactions like approaches towards odour impregnated dummies.

The standardized canonical discriminant function coefficients and factor scores of the DFA can be used to assess the importance of individual compounds in separating *Ophrys* species by pollinators. A high importance of a single hydrocarbon would show this hydrocarbon to be pollinator specific. Accordingly we found saturated and unsaturated hydrocarbons C24 to C27 to play a key role in the statistical separation of *Ophrys* by pollinator. Whether these hydrocarbons are decisive in species-specific attraction of the different pollinators remains to be proven in bioassays.

Phylogenetic relationship and convergent evolution of pollinator attracting scent. There are several cases of sympatrically and allopatrically occurring *Ophrys* species with the same pollinators (Paulus and Gack 1990a, Paulus 2001). However, it is unclear so far whether these *Ophrys* species attract pollinating males with identical compounds, and whether this represents a convergent evolution of pollinator attracting scent or is a result of the phylogenetic relationship. The solvent extracts of *O. fusca*, *O. sitiaca* and *O. herae*, all pollinated by *A. nigroaenea*, contained the same alkanes and alkenes in almost identical bouquets and released the same response in the male antennae. In our cluster analyses performed with GC-EAD active hydrocarbons or alkenes, *O. fusca, O. sitiaca* and *O. herae* always formed a common cluster, although *O. herae* belongs to the *O. mammosa*/*sphegodes* -group and is therefore less related to *O. fusca* and *O. sitiaca* than for example *O. bilunulata* and *O. israelitica* (Bateman 2001, Soliva et al. 2001).

According to current systematics (Delforge 2001), all species of the *O. fusca* group should have been placed within one cluster, independent from the pollinating bee species. Even in our cluster analyses performed with non-hydrocarbons that do not have a function in pollinator attraction (Ayasse et al. 2000), the results were not in accordance with the phylogenetic relationship. Our cluster analyses based on hydrocarbons or alkenes showed that the similarity of floral scent is in congruence with the pollinator species and not with the phylogenetic relationship. Therefore, we postulate a convergent evolution of pollinator-attracting scent as was found in the case of *O. sphegodes* and *O. fusca* (Schiestl et al. 2000, Schiestl and Ayasse 2002).

Because of this convergent evolution, chemotaxonomic investigations within the genus *Ophrys* should be preferably performed with floral volatiles that are not GC-EAD active and that do not have a function in pollinator attraction. Otherwise, the similarity of species would be dependent on their pollinator and is not based on the genetic similarity of species.

Hybrid origin of *Ophrys* species. A prezygotic isolation mechanism based on the selective attraction of males of only one pollinator species prevents pollen loss and increases

reproductive success (Ayasse et al. 2000). The investigations by Schiestl and Ayasse (2002) showed that changes in the relative proportions of only a few compounds may be sufficient to attract a new pollinator species, which could lead to the sympatric formation of a new species (Paulus and Gack 1990a) or to hybridisation, which may be another mechanism initiating speciation. Within the genus *Ophrys,* hybrid populations are well known and have been frequently described (Ehrendorfer 1980). For several species within the *O. fusca*-group a hybridogenic or introgressive origin has been proposed (Paulus 1988, Paulus and Gack 1990b), one of these is *O. sitiaca*, which was included in our study.

Ophrys sitiaca is endemic to Crete and other eastern Aegean islands and, according to Paulus (1988), the plants resemble an intermediate between *O. omegaifera* and *O. fusca*. On Crete, *O. omegaifera* occurs in sympatry with *O. sitiaca*, while the possible second parental species, *O. fusca*, does not occur there. Consequently *O. fusca* may have been displaced by *O. sitiaca*. In the cluster analyses based on all hydrocarbons, all but one specimen of *O. sitiaca* and *O. fusca* were placed in the same cluster (Fig. 3). Furthermore, our confusion matrix of the DFA indicated that one specimen of *O. fusca* should be placed in the group of *O. sitiaca*, and two specimens of *O. sitiaca* should be placed with *O. fusca*. There are two possible explanations for these patterns: 1) The similarity we found in the floral scent of *O. fusca* and *O. sitiaca* is based on a hybrid origin of *O. sitiaca*, and the two specimen have genes in common that are involved in the synthesis of the investigated scents. 2) The similarity in floral scent is based on convergent evolution of the odour signals responsible for attracting *A. nigroaenea* males. We prefer the first explanation, because *O. sitiaca* shows morphological traits and colouration of both parental species. Only an analysis using neutral genetic markers (Soliva et al. 2000, 2001; Soliva and Widmer 2003) could verify a hybrid origin of *O. sitiaca*. However, it is so far not known to what extend neutral genotypic and phenotypic variation in floral scent is correlated in orchid populations.

Conclusion and future investigations. Our investigations have shown that allopatric and sympatric *Ophrys* species with the same pollinator – independent of their phylogenetic relationship – use the same volatiles in very similar proportions for pollinator attraction. Therefore, the odour bouquet of an *Ophrys* species is under pollinator mediated stabilising selection and quantitative differences in scent between species with the same pollinator are probably driven by selection rather then genetic drift. Negative frequency dependent selection in response to odour learning in deceptive systems may favour variability of the pollinator attracting signal within orchid populations (Moya and Ackermann 1993, Ayasse et al. 2000, Gigord et al. 2001) and Ayasse et al. (2000) found considerable odour variation within *O. sphegodes* populations. This variation may cause hybridisation and speciation, if the odour might by chance resemble the sex pheromone of another species. Hybrids that attract a new pollinator that is not attracted by the parental species would successfully reproduce, being reproductively isolated from other sympatrically occurring plants, and the new odour would be established by stabilising selection. To answer the question of speciation by hybridisation, analyses of floral scent and plant material from the same individuals are needed to answer the issue of hybrid occurrence. In conclusion, future investigations with molecular techniques and chemical analyses in combination with behavioural experiments and electrophysiology will help to clarify the potential hybrid origin of *Ophrys* populations and may demonstrate processes of sympatric speciation.

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References

Ackerman J. D. (1986) Mechanism and evolution of food-deceptive pollination systems in orchids. Lindleyana 1: 108–113.

Ayasse M., Schiestl F. P., Paulus H. F., Löfstedt C., Hansson B. S., Ibarra F., Franke W. (2000) Evolution of reproductive strategies in the sexually deceptive orchid *Ophrys spehgodes*: How does flower-specific variation of odor signals influence reproductive success? Evolution 54: 1995–2006.

Ayasse M., Paxton R. J., Tengö J. (2001) Mating behavior and chemical communication in the order Hymenoptera. Annual Rev. Entomol. 46: 31–78.

Ayasse M., Schiestl F. P., Paulus H. F., Ibarra F., Francke W. (2003) Pollinator attraction in a sexually deceptive orchid by means of unconventional chemicals. Proc. Roy. Soc. London B 270: 517–522.

Bateman M. R. (2001) Evolution and classification of European orchids: insights from molecular and morphological characters. Jour. Eur. Orch. 33: 33–119.

Baumann H., Künkele S. (1982) Die wildwachsenden Orchideen Europas. Kosmos Naturführer. Franckh'sche Verlagshandl., Stuttgart.

Baumann H., Künkele S. (1986) Die Gattung *Ophrys* L. - eine taxonomische Übersicht. Mitt. Bl. Arbeitskr. Heim. Orch. Baden-Württ. 18: 305–688.

Baumann H., Künkele S. (1988) Neue Beiträge zur Taxonomie europäischer und mediterraner Orchideen. Mitt. Bl. Arbeitskr. Heim. Orch. Baden-Württ. 20: 610–651.

Bergström G. (1978) Role of volatile chemicals in *Ophrys*-Pollinator interactions. In: Harborne G. (ed.) Biochemical aspects of plant and animal coevolution. Academic Press, pp. 207–230.

Borg-Karlson A.-K. (1990) Chemical and ethological studies of pollination in the genus *Ophrys* (Orchidaceae). Phytochemistry 29: 1359–1387.

Borg-Karlson A.-K., Bergström G., Groth I. (1985) Chemical basis for the relationship between *Ophrys* orchids and their pollinators. 1. Volatile compounds of *Ophrys lutea* and *O. fusca* as insect mimetic attractants/excitants. Chemica Scripta 25: 283–311.

Borg-Karlson A.-K., Bergström G., Kullenberg B. (1987) Chemical basis for the relationship between *Ophrys* orchids and their pollinators. 2. Volatile compounds of *Ophrys insectifaera* and *O. speculum* as insect mimetic attractants/excitants. Chemica Scripta 27: 303–311.

Buser H.-R., Arn H., Guerin P., Rauscher S. (1983) Determination of double bond position in mono-unsaturated acetates by mass spectrometry of dimethyl disulfide adducts. Anal. Chem. 55: 818–822.

Dafni A. (1984) Mimicry and deception in pollination. Annual Rev. Ecol. Syst. 15: 259–278.

Delforge P. (2001) Guide des Orchidees d'Europe, d'Afrique du Nord et du Proche-Orient. Delachaux et Niestlé S.A., Lausanne-Paris.

Dunkelblum E., Tan S. H., Silk P. J. (1985) Double-bond location in mono-unsaturated fatty acids by dimethyl disulfide. Derivatization and mass spectrometry: Application to analysis of fatty acids in pheromone glands of four Lepidoptera. J. Chem. Ecol. 11: 265–277.

Ehrendorfer F. (1980) Hybridisierung, Polyploidie und Evolution bei europäisch-mediterranen Orchideen. Die Orchidee Sonderheft: 15–34.

Foelsche G., Foelsche W. (2001) *Ophrys africana* spec. nov., ein früh blühendes Taxon der *Ophrys fusca*-Gruppe in Tunesien. Jour. Eur. Orch. 33: 637–672.

Foelsche G., Foelsche W. (2004) Anmerkungen und Berichtigungen zu Publikationen über *Ophrys gazella*, *Ophrys africana*, *Ophrys corsica* und *Orchis corsica*. Jour. Eur. Orch. 36: 1009–1024.

Gigord L. D. B., Macnair M. R., Smithson A. (2001) Negative frequency-dependent selection maintains a dramatic flower colour polymorphism in the rewardless orchid *Dactylorhiza sambucina* (L.) Sóo. Proc. Natl Acad. Sci. USA 98: 6253–6255.

Ibarra F. (2002) Intra- und interspezifische chemische Kommunikation von Insekten. Identifizierung und Synthese flüchtiger Signalstoffe. Dissertation, Universität Hamburg.

Kullenberg B. (1961) Studies in *Ophrys* pollination. Zool. Bidr. Upps. 34: 1–340.

Kullenberg B. (1973) New observations on the pollination of *Ophrys* L. (Orchidiaceae). Zoon (Suppl. 1): 9–13.

Kullenberg B., Bergström G. (1976) The pollination of *Ophrys* orchids. Bot. Notiser 129: 11–19.

Mc Lafferty F. W., Stauffer D. B. (1989) The Wiley NBS Registry of Mass Spectral Data. Wiley Interscience, New York.

Moya S., Ackermann J. D. (1993) Variation in the floral fragrance of *Epidendrum ciliare* (Orchidaceae). Nord. J. Bot. 13: 41–47

Nilson A. (1992) Orchid pollination biology. Trends Ecol. Evol. 7: 255–259.

Paulus H. F. (1988) Beobachtungen und Experimente zur Pseudokopulation auf *Ophrys*-Arten (Orchidaceae) Kretas (II) mit einer Beschreibung von *Ophrys sitiaca* H.F. Paulus & C. + A. Alibertis nov. spec. aus dem *Ophrys fusca-omegaifera*-Formenkreis. Mitt. Bl. Arbeitskr. Heim. Orch. Baden-Württ. 20: 817–882.

Paulus H. F. (2001) Material zu einer Revision des *Ophrys fusca* s.str. Artenkreises I. – *Ophrys nigroaenea-fusca*, *O. colletes-fusca*, *O. flavipes-fusca*, *O. funera*, *O. forestieri* oder was ist die typische *Ophrys fusca* Link 1799 (Orchidaceae)? Jour. Eur. Orch. 33: 121–177.

Paulus H. F., Gack C. (1986) Neue Befunde zur Pseudokopulation und Bestäuberspezifität in der Orchideengattung *Ophrys* – Untersuchungen in Kreta, Süditalien und Israel. Die Orchidee Sonderheft: 48–86.

Paulus H. F., Gack C. (1990a) Pollinators as prepollinating isolation factors: evolution and speciation in *Ophrys* (Orchidiaceae). Israel J. Bot. 39: 43–79.

Paulus H. F., Gack C. (1990b) Pollination of *Ophrys* in Cyprus. Pl. Syst. Evol. 169: 177–207.

Paulus H. F., Gack C. (1994) Signalfälschung als Bestäubungsstrategie in der mediterranen Orchideengattung *Ophrys* – Probleme der Artbildung und der Artabgrenzung. Eurochris 92: 45–71.

Peakall R. (1990) Responses of male *Zaspilothynnus trilobatus* turner wasps to female and the sexually deceptive orchid it pollinates. Funct. Ecol. 4: 159–167.

Pijl van der L., Dodson C. H. (1966) Orchid flowers: their pollination and evolution. Univ. of Miami Press, Coral Gables, FL.

Pouyanne A. (1917) Le fecondation des *Ophrys* par les insects. Bull. Soc. Hist. Nat. Afr. Nord 8: 6–7.

Renz J. (1929) Über neue Orchideen von Rhodos, Cypern and Syrien. Repert. Spec. Nov. Regni. Veg. 27: 193–218.

Schiestl F. P., Ayasse M. (2001) Post-pollination emission of a repellent compound in a sexually deceptive orchid: a new mechanism for maximizing reproductive success? Oecologia 126: 531–534.

Schiestl F. P., Ayasse M. (2002) Do changes in floral odor cause speciation in sexually deceptive orchids? Pl. Syst. Evol. 234: 111–119.

Schiestl F. P., Marion-Poll F. (2002) Detection of physiologically active flower volatiles using gas chromatography coupled with electroantennography. In: Jackson J. F., Linskens H. F., Inman R. B. (eds.) Molecular methods of plant analysis 21. Springer, Berlin Heidelberg, pp. 173–198.

Schiestl F. P., Ayasse M., Paulus H. F., Löfstedt C., Hansson B. S., Ibarra F., Francke W. (1999) Orchid pollination by sexual swindle. Nature 399: 421–422.

Schiestl F. P., Ayasse M., Paulus H. F., Löfstedt C., Hansson B. S., Ibarra F., Francke W. (2000) Sex pheromone mimicry in the early orchid (*Ophrys sphegodes*): patters of hydrocarbons as the key mechanism for pollination by sexual deception. J. Comp. Physiol. A 186: 567–574.

Schmid-Egger C., Scheuchl E. (1997) Illustrierte Bestimmungstabellen der Wildbienen Deutschlands und Österreichs unter Berücksichtigung der Arten der Schweiz. Eigenverlag, Velden/Vils.

Singer R. B. (2002) The pollination mechanism in *Trigonidium obtusum* Lindl. (Orchidaceae: Maxillariinae): Sexual mimicry and trap-flowers. Ann. Bot. 89: 157–163.

Soliva M., Widmer A. (2003) Gene flow across species boundaries in sympatric, sexually deceptive *Ophrys* (Orchidaceae) species. Evolution 57: 2252–2261.

Soliva M., Gautschi B., Salzmann C., Tenzer I., Widmer A. (2000) Isolation and characterisation of microsatellite loci in the orchid *Ophrys araneola* (Orchidaceae) and a test for cross-species amplification. Molec. Ecol. 9: 2178–2179.

Soliva M., Kocyan A., Widmer A. (2001) Molecular phylogenetics of the sexually deceptive orchid genus *Ophrys* (Orchidaceae) based on nuclear and chloroplast DNA sequences. Molec. Phylogent. Evol. 20: 78–88.

SPSS. (1997) SPSS base 7.5 for Windows user's guide. SPSS Inc. Chicago.

Steiner K. E., Whitehead V. B., Johnson S. D. (1994) Floral and pollinator divergence in two sexually deceptive South African orchids. Amer. J. Bot. 81: 185–194.

Tengö J. (1979) Odour-released behaviour in *Andrena* male bees. Zoon 7: 15–48.

Westrich P. (1989) Die Wildbienen Baden-Württembergs I, II. Eugen Ulmer Verlag, Stuttgart, Germany.

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