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Deceived males – Pollination biology of the Mediterranean orchid genus *Ophrys* (*Orchidaceae*)

Keywords

Pollination biology, *Ophrys*, *Orchidaceae*, *Insecta*, *Hymenoptera*, *Apoidea*, pseudocopulation, sexual deception mechanisms, learning behaviour of pollinator, chemical ecology.

Summary

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The flowers of the Mediterranean orchid genus *Ophrys* imitate important highly specific sexual releasing factors of aculeate hymenopteran females. They attract female seeking males in a species-specific manner. The males attempt to copulate with the labellum of the flower. During this pseudocopulation the males remove the pollinia. If the male is attracted to another flower of the same *Ophrys* species and if sexual mimicry is successful again, that flower can become pollinated.

- Each *Ophrys* species has its own pollinator species. Since the relationship is species-specific, the pollinators serve as a pre-pollinating isolation factor. The flowers benefit from the pre-mating isolation factors of their pollinator species to ensure their own genetic isolation from similar *Ophrys* species.
- This is illustrated in numerous examples and demonstrated in many experimental choice-tests in the field under natural conditions.
- Evolution of new *Ophrys* species is always a consequence of a change in pollinator. The new pollinator-species selects those individual flowers for pollination which come closest to its own female's releasing stimuli and consequently selects for that stimuli.
- If the systematic status of an *Ophrys* species is unclear, discovery its own pollinator provides evidence of its biospecies status.

- A system where the same species pollinates different *Ophrys* species is possible only if the plants in question are distributed allopatrically or syntopically, or if they are visited by bees which differ in the location where the pollinia are attached, i.e., head or abdomen. Examples for both types are given.
- In such a system the appearance of the flowers of the different *Ophrys* species can be very similar. This convergence or parallelism occurs as a result of identical selection.

In several investigations we found that the *Ophrys* flower produce exact the same chemical compounds as these of the pollinator female to attract a male. To maximise pollination success *Ophrys* flowers makes use of the learning behaviour of the pollinator males. Presenting besides the specific compounds they produce many further compounds to have an individuality. Deceived males recognise these and avoid just visited flowers like unattractive females. They were called to another flower. This behaviour prevents selfpollination and maximise allogamy. Another trick is to produce after pollination a so-called antiaphrodisiacum which signals that the flower is already pollinated like in their own females which signals they are mated and not longer attractive.

Zusammenfassung

Paulus, H.F. (2006): Getäuschte Männchen – Bestäubungsbiologie der mediterranen Orchideengattung *Ophrys* (*Orchidaceae*). - Jour. Eur. Orch. 38(2): 303-353.

Blüten der mediterranen Orchideengattung *Ophrys* imitieren alle wichtigen, Paarung auslösenden Signale der Weibchen aculeater Hautflügler (Hymenoptera). Sie locken damit Weibchen suchende Männchen in artspezifischer Weise an. Diese Männchen versuchen dann mit der *Ophrys*-Blüte zu kopulieren. Während dieser Pseudokopulation entnehmen sie die Pollinarien. Wird dieses Männchen von einer weiteren *Ophrys*-Blüte angelockt, die Weibchen-Mimikry erneut erfolgreich war, wird diese bestäubt.

- Jede *Ophrys*-Art hat ihren eigenen Bestäuber. Da diese Beziehung artspezifisch ist, fungiert der Bestäuber als Präpollinations-Isolationsmechanismus. Die Blüten profitieren dadurch, dass ihr Pollen nur innerhalb der Art transferiert wird. Der Bestäuber sorgt für die genetische Isolierung der Art.
- In zahlreichen Freilanduntersuchungen im gesamten Mittelmeergebiet konnte dies durch Beobachtungen und vor allem durch experimentelle Wahltests in den Habitaten bestätigt werden.
- Die Entstehung neuer Arten ist daher eine Konsequenz eines Wechsels des Bestäubers. Der neue Bestäuber selektiert zwischen solchen *Ophrys*-

Individuen, die seiner Weibchen-Erwartung näher kommen und verhilft dadurch nur diesen Individuen zur Samenproduktion.

- Diese enge Beziehung ist andererseits ein sehr gutes Hilfsmittel, um unklare Sippen auf ihren Artstatus zu überprüfen.
- Arten mit derselben Bestäuberart können nur allopatrisch verbreitet sein oder sie bestäuben syntope Arten einmal per Kopf-, die andere per Abdomenpollination.

In einigen Untersuchungen konnten wir zeigen, dass die Blüten im Vergleich mit den Weibchen mit chemisch identischen Duftbouquets die Bestäubermännchen anlocken. Zur Bestäubungsmaximierung nutzen die Blüten das Lernvermögen der Bestäuber, um durch ihre individuelle Duftnote und individuelle Blütenmuster den einmal getäuschten Männchen das Wiedererkennen zu ermöglichen. Sie meiden solche einmal besuchten Blüten, da sie diese für unattraktive Weibchen halten. So werden sie auf neue Blüten gelenkt. Darüber hinaus signalisieren sie sogar, dass sie bereits bestäubt sind (Antiaphrodisiakum). Genau dies tun nämlich auch ihre Weibchen, um nicht länger belästigt zu werden.

* * *

1. Introduction

The Orchid species belonging to the Mediterranean genus *Ophrys* have been long known for their bizarre and exotic appearance. To some extent the flower resembles an insect or spider and because of this they have been labeled bee orchids, bumblebee orchids, spider orchids and fly orchids, just to mention the most common Central European names.

The biological significance of the flower's appearance remained entirely hidden for a long time. Even one of the most experienced authorities on the pollination biology of orchids, the evolutionist CHARLES DARWIN, managed to devote only a few lines to the genus *Ophrys* in his book "On the various contrivances by which British and foreign orchids are fertilised by insects..." (1862). He cited the rather peculiar observations of a Mr. Price, who maintained that bees attacked the flowers and treated them like little devils that had to be defeated. DARWIN admitted that he was not able to make any sense out of this behavior. That a grain of truth lay hidden in the apparent attacks is something that DARWIN could not have suspected. The secret behind the unusual appearance of the flowers of *Ophrys* was first actually recognized by the Frenchman POUYANNE working in Algeria. In the year 1916, he published an article in French in the Journal of the French Society of Horticulture entitled

“A curious case of mimicry in *Ophrys*”. He noted that the flowers of the mirror orchid *Ophrys vernixia* (= *speculum, ciliata*) were visited only by the solitary wasp *Dasyscolia ciliata* (formerly called *Campsoscolia*) (Hymenoptera, Scoliididae) which resembles somewhat the appearance of a bee. Further, only male wasps visited the flower and they performed movements on the lip of the flower which clearly seemed to be attempts to copulate with it. Since, in no instance were they obtaining nourishment, he logically inferred that the males acted as if the *Ophrys* flower as one of their own females. Understandably, scarcely anyone in the scientific community believed him at first. But Pouyanne, in fact, must be credited with discovering a new form of pollination which at the same time is also a new form of mimicry. It was named in his honor - Pouyanne mimicry, but is more commonly known as “pseudocopulation” (CORREVON & POUYANNE 1916, 1923, POUYANNE 1917; GODFERY 1925, 1929). Later, KULLENBERG’s (1961) extensive study brought clarification and credibility to the phenomena. He suggested that the *Ophrys* flowers must mimic in large part the female sexual pheromone of the pollinators and thereby trick the males into visiting the flowers which would ultimately promote pollination.

All species of *Ophrys* achieve pollination by means of sexual deception, and the behavior displayed by the males is called pseudocopulation. Only two species have hit upon different strategies of pollination: *O. apifera* which is an obligate self-pollinator and *O. helenae* which attracts pollinators in an even more unusual manner, namely by roost-mimicry. It may also be called sleeping-hole-mimicry, since male bees of genera *Tetralonia* and *Eucera* (Eucerini) are lured to the flowers evenings when they search for an overnight sleeping site (PAULUS & GACK 1993). The Japanese orchid *Cymbidium pumilum* apparently also engages in a form of roost-mimicry with the drones of the Asian honeybee *Apis cerana japonica* (SASAKI et al. 1991). The orchid produces a fragrance, in other words an olfactory stimulus, which imitates the aggregation-pheromone of the bees. It might also be mentioned that pollination by sexual deception also occurs in 11 genera of Australian orchids (COLEMAN 1927, STOUTAMIRE 1975, BEARDSELL & BERNHARDT 1982, SCHIESTL et al. 2003), and in the South American group of *Maxillariinae* (*Trigonidia*, *Mormolyca* which are pollinated by drones of stingless bees: *Meliponinae*: SINGER 2002, SINGER et al. 2004) and *Lepanthes* (*Pleurothallidinae*, pollinated by males of fungus gnaths, *Sciaridae*: BLANCO & BARBOZA 2005).

A main questions arise regarding *Ophrys* and its practice of sexual deception: How do flowers create a pollination system based on sexual deception?

One plausible assumption was that the flowers imitate precisely those signals which release male mating behavior, whereby the pollinator male acts as if the flower were a conspecific female and answers with copulatory attempts. The

signals which do this kind of effect in males are known from numerous other insects and generally include the visual, olfactory and tactile stimulus. In addition, the necessary sensory apparatus must be developed in the receiver, in our case it is the males, which will be manipulated. How the behavioral release mechanisms operate at the receptor and the neural levels is known only in few cases. In addition, field experiments must demonstrate that the attractiveness of the *Ophrys* flowers is actually species specific.

In extensive and long-term field studies, PAULUS and coworkers have consistently shown in choice-tests that each species of *Ophrys* has only one effective pollinator. Occasionally other flower-visitors are observed, they however are either very closely related species or they were apparently lured for unknown reasons to the flower. These accidental pollinators can be responsible for a small proportion of pollinations in some of the years. The small list of known pollinators at the time of KULLENBERG (1961) has considerably increased in later publications (PAULUS & GACK 1990a, 1994). In the process many previously unknown species were discovered and their species status could be demonstrated by means of biotests (e.g. PAULUS 1998, 2001). A biological species concept was developed which postulated that formation of a new *Ophrys* species must occur with the acquisition of a new pollinator (PAULUS & GACK 1983, 1990a). Through a strict and consistent application of this concept, the number of recognized species in the genus increased from original estimates of 30 to 40 species to far over 250 (DELFORGE 2005). This species concept has also been successfully applied to the Australian sexually deceptive flowers (BOWER & BROWN 1997). Based on currently known information, I will show how *Ophrys* flowers achieve sexual deception, what the differences are between the species in this respect and how this may influence evolution and the formation of new species. A further point of interest, which in the meantime we better understand, are the various methods of maximizing pollination within the framework of sexual selection.

Table 1

Pollinators of the genus OPHRYS (ca. 260 species); * = abdominal pollination

Pollinator	Ophrys species group	Number of species	Distribution
Scoliidae	<i>vernixia</i>	1	Mediterranean Area
Sphecidae	<i>insectifera</i>	1	Central Europe
Argidae	<i>subinsectifera</i>	1	N-Spain
<u>Apoidea, Colletidae</u>			
Colletes	<i>exaltata-arachnitiformis</i>	2-7	Centr. Mediterranean: NE-Spain to Dalmatia and NW-Greece, Ionian Islands
Colletes	* <i>murbeckii</i>	1	Tunesia, E-Algeria
Colletes	* <i>algarvensis</i>	1	S-Spain, S-Portugal
<u>Apoidea, Andrenidae</u>			
Andrena	<i>spegodes</i> -group	ca. 65	Europe, Mediterranean Area
Andrena	* <i>fusca-lutea</i> -aggr.	ca. 65	Mediterranean Area
Andrena	<i>aymoninii</i>	1	S-France
<u>Apoidea, Anthophoridae</u>			
Anthophora	* <i>omegaifera-dyris</i> -group	4-6	Western/East. Mediterr.
Anthophora	<i>argolica-crabronifera</i> -group	9	Central/eastern. Mediterranean Area
Melectini	<i>cretica-reinholdii</i>	6	S-Greece, Turkey
Xylocopa	<i>spruneri, grigorigiana</i>	2	Greece, Crete
	<i>sipontensis</i>	1	S-Italy
Eucera/ Tetraloniella	<i>holoserica-scolopax-oestrifera</i> -group	ca. 70-80	Mediterranean Area, Central-Europe
<u>Apoidea, Megachilidae</u>			
Osmia	<i>lunulata, promontorii, tarentina, panattensis</i>	4	Italy, Sardinia, Sicily
Chalicodoma	<i>bertolonii-bertoloniiiformis-melitensis</i> -group	10	S-France, N-Spain, Italy, Balearic Islands, Croatia, Malta
Chalicodoma	<i>ferrum-equinum</i>	2-3	Greece, Turkey
Chalicodoma	<i>gottfriediana</i>	1	Kephallonia
Chalicodoma	* <i>atlantica</i>	1	S-Spain, N-Africa
<u>Coleoptera, Scarabaeidae</u>			
Blitopertha	* <i>blitopertha</i>	2	SE-Greece, Turkey

2. The principle of *Ophrys* pollination

The regular pollinators of *Ophrys* species, as known so far, are only males of aculeate *Hymenoptera* and two species of beetles from the genus *Blitopertha* (*Scarabaeidae*). *Blitopertha lineolata* pollinates *Ophrys blitopertha* on southeastern Aegean islands, and *B. majuscula* pollinates *O. urteae* in southern Turkey (PAULUS & GACK 1990c and unpubl.) In rare cases however the beetle *Phyllopertha horticola* and the hoverfly *Microdon* (ENGEL 1985, many pers. obs.) may serve as additional and therefore secondary pollinators of *Ophrys holoserica* (Central Europe). Presumably, a part of the odor bouquet appeals to the males of these species (BORG-KARLSON 1989). Furthermore, it is possible that another hoverfly species, *Merodon velox*, is the pollinator of *O. regis-ferdinandii* on eastern Aegean islands. In this case, however, the flowers mimic male hoverflies which releases aggressive behavior in the males that are territorial (PAULUS & GACK 1990c). The flowers conspicuously resemble the strikingly coloured males of these *Syrphidae*. Finally, the completely featureless labellum pattern of the flowers of the Greek species *Ophrys helenae* imitates a dark hole. Evenings, the males of several closely related Eucerini species, especially *Eucera (Synhalonia) lucasi*¹ and *berlandi*, attempt to crawl into the “mimic of a hole” to overnight (mimicry of sleeping holes). The signals which are responsible for achieving the attractiveness have not yet been closely examined. The working hypothesis is that, in addition to the optical signal for a black “hole”, the male aggregation pheromone may also play a role (PAULUS & GACK 1993). Numerous male bees in the early evening seek out a sleeping site which may be a hole in the ground or a flower which closes at night. Quite often males prefer to sleep in the company of conspecifics, thus we can assume that an olfactory orientation should be present. With few exceptions, it is primarily species from various families of bees that act to transfer the pollinia (packets of pollen). It must also be noted that *Ophrys vernixia* is pollinated by the solitary wasp *Dasyscolia (Campsoscolia) ciliata* (*Scoliidae*) (POUYANNE 1917, KULLENBERG 1949, 1961; GÖLZ & REINHARD 1977, PAULUS 1978). In addition, the flowers of *Ophrys insectifera* are pollinated by the digger wasp *Argogorytes mystaceus* and to a lesser extent also by *A. fargei* (*Sphecidae*) (WOLF 1950, KULLENBERG 1956). Probably, *Ophrys cilicica* in southeastern Anatolia is also pollinated by *Argogorytes* (PAULUS & GACK 1990a). The bees which pollinate *Ophrys* flowers include, above all, the *Andrenidae* with numerous species in the genus *Andrena* (*O. fuscilutea* aggr., *O. sphegodes* aggr.). In addition, there are three species of *Colletes* (*Colletidae*), and several species from the following groups of *Apidae*: *Anthophora* (*Anthophorini*), the cleptoparasitic taxa *Melecta* and *Eupavlovskia*

¹ formerly placed in a separate genus *Tetralonia lucasi* or *Tetralonia berlandi*

(*Melectini*), the *Eucerini* genera *Eucera* (s. lat.) and *Tetraloniella* (*O. holoserica* s. lat. aggr.), the carpenter bees *Xylocopa iris* (*O. sipontensis*, *O. spruneri*) and *X. violacea* (*O. grigoriana*²) (PAULUS in prep.) as well as the socially parasitic bumblebee *Bombus* (*Psithyrus*) *vestalis* (*O. chestermanii* and *O. normanii*, Sardinia, PAULUS & GACK 1995). Current summaries of pollinator species are found in PAULUS & GACK (1986, 1990a, b, c, d; 1995). Besides all these regular pollinators there are a number of insects which are attracted more or less accidentally. Most of them are just visitors because normally they are not able to pollinate. Some good examples are figured by BERGER (2003).

Flower visitation almost always proceeds according to a particular scheme. When a mate-seeking male of a pollinator species is underway and encounters a trace of odor emitted by an *Ophrys* flower, he begins flying in a search pattern aiming toward the source of the odor. According to field observations this occurs at a distance of approximately five to ten meters.

The search flight-pattern is characterized by a more or less zigzag path which begins to close in on the flower at about one to two meters before it. As soon as the male sees the apparent female, he rushes in at the flower, lands, adjusts to the proper seating position and begins with vehement efforts to copulate. Often the genital apparatus has already been extended before landing. The male tries again and again, probing and poking with his abdomen (= metasoma) to find the genital opening of the pseudofemale and he emits loud buzzing sounds. After a moment he sits quietly, then with emphatic wing buzzing he renews the attempt to stimulate the pseudofemale. With these violent movements the male very quickly comes into contact with the sticky disc of the pollinium, which is pulled out of its slot. Generally, the male flies off after 10 to 30 seconds. He then very often lands on another flower and the whole sequence are played out again except he usually terminates it sooner.

What significance can be ascribed to the individual components of behavior has not even been determined for the normal mating behavior of pollinator species. The fact that the approach flight follows a zigzag pattern does not infer the presence of a concentration gradient. This flight pattern enables the males to remain on an odor trail and focus in on the source. From a physio-chemical point of view, BALKOVSKY & SHRAIMAN (2002) have recently shown that odor trails can be complex structures which can be conceptualized as small discontinuous pockets of dispersing materials that are likely to be moving in a random direction with respect to mean air motion. Thus there is probably no

² From investigation of the type material of *O. sphaciotica* this taxon belongs to *O. spruneri* and not to *O. grigoriana*. That means the correct name of the large *spruneri* type of Crete is *O. grigoriana*.

continuous gradient of concentration which males follow. Vigorous wing buzzing and periodic thorax (= mesosoma) vibration occur in all species of pollinating bees. As far as known the same components of behavior are also widespread in proper mating systems (ALCOCK et al. 1978, EICKWORT & GINSBERG 1980). These behaviors are very conspicuous in *Osmia rufa* (RAW 1976, MADDOCKS & PAULUS 1987), which is the pollinator of *Ophrys panattensis* on Sardinia (PAULUS & GACK 1995). Here, in the context of sexual selection the vibration signal can presumably be used by the females to estimate the body size of the male and thus as an indicator of male fitness (LARSEN et al. 1986, SEIDELMANN 1999). Conclusive studies however have not been conducted. Very often during pseudocopulation males repeatedly attempt to induce a reaction in the apparent female through vehement wing buzzing in combination with thorax vibration. Since the pollinia are often removed immediately after landing on the surface of the lip of the *Ophrys* flower, the orchids have invented a simple trick to avoid self-pollination during the continued copulatory attempts on the same flower. At first, the removed pollinia retain a vertical position for about one to two minutes, after which the stalk begins to sink forward due to a drying process. Not until this has completed are the pollinia capable of accomplishing pollination. The males can thus without danger of causing self-pollination fiddle about on the same flower or plant, fly off and even return to it. Already DARWIN (1862) had meticulously examined the sinking of the pollinia in various orchids. He recognized that the time required for complete descend of the pollinia, for example in *Orchis mascula*, corresponded exactly with the average time needed for a bumblebee to visit the next inflorescence. The pollinia adhere firmly to the head or abdomen, despite vigorous grooming attempts the bees cannot remove them. Such males are spoken of as “marked” and they carry the pollinia for several days or even weeks. Just before flying off males may groom their antennae by pulling them through the cleaning structure on the forelegs. After that, many bees particularly the Eucerini will hover over the flower for a few seconds. Is the bee examining the flower and its odor again and going through a learning process? They almost never revisit the same flower again but seek out new flowers. This may be due to their ability to individually recognize those flowers which they have already encountered. In fact, we could show that some of the deceived pollinator males were, so to say, able to soon see through the fraud, and they no longer fell for the dummy female (PAULUS et al. 1983, PAULUS & GACK 1984, PAULUS 1988a). At first, males will avoid the individual plants which they have already visited, and later they cease to visit any *Ophrys* flowers at all. The flowers obtain new visitors almost always from flower-naive males. This may help explain why the phenomenon of pseudocopulation was previously rarely observed. The likelihood of being present when a pseudocopulation actually occurs is very low considering that it

may only occur only once during the entire flowering period of a plant. An individual plant blooms for about three to four weeks. The rate of pollination in an *Ophrys* population hardly ever exceeds 5 to 10%. Nonetheless, this rate of pollination is more than enough to ensure the survival of the population. Even if pollination seldom occurs, it is still highly effective. Each pollinated flower may yield over 30,000 seeds (NAZAROV & GERLACH 1997). The Central European species *Ophrys holoserica* and *O. sphegodes* contain approximately 12,000 to 14,000 seeds per flower (PAULUS, unpubl. data). We now turn to the tricks which *Ophrys* flowers employ to deceive males and how they achieve this on a species-specific level.

2.1. Methods of signal forgery

In order to achieve pollination, the flowers of the species of the orchid genus *Ophrys* imitate the signals which release mating behavior in the pollinator species (KULLENBERG 1961, KULLENBERG 1973 a, b, PAULUS & GACK 1980, 1990b). These signals developed primarily in the insect pollinator through of its own sexual evolution. They serve as well-functioning mechanisms for recognition of species and thus for prevention of hybridization. In addition they may play an important roll in the context of mate selection. Three basic sets of signals or stimuli are critical here – olfactory, visual and tactile.

Each set represents a more or less complex pattern having the attributes of key stimuli and are therefore regarded as innate releasing mechanisms. It is known from the reproductive biology of many insects that females produce chemical attractants (sexual pheromone) to lure a sexual partner. The olfactory receptors on the male antennae are able to perceive the pheromone, and the male responds by searching for the female. The males of each species have evolved their own innate releasing mechanisms which enable them to pick exactly that blend which their females emit from thousands of possible odor-stimuli. Once a male is in the near vicinity of a female, he is able to visually perceive the optical signals given off by the female which are species specific. After the male lands on the female, the third set of stimuli, namely tactile, comes into action. With the help of this information, the male is able to determine which end of the female body is the front and which is the back. For this the direction of the female's body hairs are registered (KULLENBERG 1960, PIRSTINGER 1996). The males must perform this task quickly because of the presence of competing males with the same intention; whoever is the fastest win (scramble competition). To what extent the tactile stimuli are species-specific has not been examined. Up to now, experiments and comparative scanning electron microscope (SEM) studies on the hairs of the labellum of the flower have not yielded sufficient evidence (PIRSTINGER 1996). It is possible that species-

specific tactile stimuli only occur in connection with physical contact of chemical substances. It will be shown in the following that *Ophrys* flowers are able to imitate all of these signals and thereby successfully attract particular species of pollinator males, deceive them and profit from the transfer of the pollinia.

2.1.1. Olfactory signals

In glandular cells under the labellar surface of each *Ophrys* flower, hundreds of odor-components are produced which according to their mixture and concentration make up the species-specific odor-bouquet (perfume). The chemical substances include, above all, terpenoids, long-chained aliphatic hydrocarbons, aldehydes, ketones and 1-, 2-alcohols as well as cyclic (aromatic) compounds. All of these substances are commonly used as scents and fragrances in the plant and animal kingdoms. Since 1978, BERGSTRÖM, HEFETZ, BORG-KARLSON and others have documented the chemistry of the olfactory attraction based on analyses of flower and bee odors. Summaries for insects are given in ROELOFFS (1995), FRANCKE & SCHULZ (1999), for bees in DUFFIELD et al. (1984), FREE (1987), ENGELS et al. (1997), and most currently in AYASSE et al. (2001). However, it was difficult for a long time to make any sense out of the astoundingly long list of volatile odor compounds present in *Ophrys* flowers (over 100 different volatiles), especially since no agreement was found in qualitative studies of the odor-chemistry between the flower and the pollinator (BORG-KARLSON 1987, 1990). Behavioral experiments utilizing synthetic copies of compounds produced by *Ophrys* flowers have shown that only certain volatiles are active in stimulating mating behavior in males (KULLENBERG & BERGSTRÖM 1976, TENGÖ 1979). BORG-KARLSON (1990) concluded that *Ophrys* flowers produce a set of “second-class” attractant compounds that lure only that small part of the pollinator population with a low threshold for sexual stimuli during the period when females have not yet appeared. This led to a long-standing assumption about odor mimicry whereby the flowers simply make use of *different* chemicals.

Lists of the odor components obtained from *Ophrys* flowers by gas chromatography were provided first by BERGSTRÖM (1978) and later especially by BORG-KARLSON (1987, 1990) and BORG-KARLSON et al. (1985, 1987). They showed that all species, which are recognized in accordance with proper systematic treatment, have their own highly specific blend of odors. It is remarkable that these mixtures are never found in the fragrance produced by the female of the pollinator and, if so, they agree only in single components. It must be noted that these investigations failed to comparatively examine the appropriate objects, namely virgin females (BERGSTRÖM & TENGÖ 1974, 1978, BERGSTRÖM et al. 1982, FRANCKE et al. 1981, 1984, TENGÖ & BERGSTRÖM 1976,

1977, BORG-KARLSON 1990, etc.). Thus until recently we have known actually very little about the male stimulating components. From the long list of volatile odor compounds which have been discovered nothing matches the species-specific components which are responsible for attracting the males. One of the first investigations of male reaction using electroantennographic detection was conducted by PRIESNER (1973). He showed that the antennae of male *Andrena* bees were able to respond to mixtures of odor produced by *Ophrys* flowers. However, those species which pollinate *Ophrys* flowers unfortunately were not investigated so that we cannot draw any further conclusions from the results. Up until this point, it was assumed that *Ophrys* species achieve odor mimicry by provoking the same physiological and behavioral reactions in the males which their females also release, however, using a different chemistry (KULLENBERG 1973A, STOWE 1988). Based on numerous field observations it becomes obvious that innate behavior mechanisms of males are released by the copying signals and phony female scents of *Ophrys* flowers, just as if they were genuine female bee stimuli. Furthermore, we disproved in numerous field tests the hypothesis that the *Ophrys* flowers only elicit a reaction as long as the females are not yet actively flying. Our experiences show that the *Ophrys* flowers will always excite males even long after the females have emerged (PAULUS 1988, PAULUS & GACK 1990).

Is the odor bouquet an equivalent or a perfectly copied signal?

Since previous attempts have failed to identify which of the many hundreds of volatile odor compounds cause the male to react as if they were emitted from an unmated female, an entirely new procedure was called for. First, it was necessary to determine the composition of the sexually attractive scent of the female; otherwise it would not be clear what to search for. This task was aggravating because no sexual pheromone had yet been isolated from any species of solitary bees. Analysis of female odor resulted in an unusually large accumulation of primarily long-chained hydrocarbons; however, it could not be assessed which volatiles and which blends actually function as the sexual pheromone.

Using a combination of methods: gas chromatography plus electroantennographic detection (GC-EAD), gas chromatography plus mass spectrometry (GC-MS), as well as behavioral field tests, we could identify compounds that mediate male behavior in the orchid *Ophrys sphegodes* and its pollinator bee *Andrena nigroaenea* (SCHIEL et al. 1999, 2000) as well as in *Ophrys speculum* (= *vernixia*) and its pollinator flower wasp *Dasyscolia (Campsoscolia) ciliata* (AYASSE et al. 2003). Furthermore, we tested the hypothesis that *Ophrys* flowers produce only “second class” attractant compounds (BORG-KARLSON 1990) that

are less attractive than the genuine females.

This could only be shown to be false through the combination of gas chromatographic analysis of the odor along with an electrophysiological action potential of the whole antenna for the reactions of the receptors in the male. To accomplish this, the fragrance of the orchid *Ophrys sphegodes* and its pollinator *Andrena nigroaenea* were examined. First, all volatile odor compounds emitted from the flower's labellum were characterized and compared with the chemical components washed off the cuticula (cuticular wash) of virgin *Andrena nigroaenea* females. The individual components of both blends were identified using gas chromatography. Each type of odor volatile was tested for an electrophysiological reaction (action potential) on male antennae. The results showed that the receptors on the antennae of the males responded to 16 odor volatiles found in both the flower and the female scents. In subsequent behavioral tests (biotests) a bouquet of exactly these 16 volatile odor compounds was presented to males flying outdoors. They reacted to this mixture with specific copulation movements (SCHIELTL et al. 1999, 2000). The chemical composition of the male attractants was thus demonstrated for the first time along with the fact that the attractants of the flower and those of the female were completely identical. The *Ophrys* system of mimicry operates not because the chemistry is roughly equivalent but because the species-specific stimuli of the female scent are precisely identical. Apparently, many of the closely related *Ophrys* species utilize species-specific blends of the same hydrocarbons. The same principle of species-specific mate attraction also applies to various species of moths (ROELOFFS 1995). The moths are closely related and sympatrically distributed, like many *Ophrys* species. The necessary species-specificity of the blends is easily achieved by varying the relative proportion of common compounds. The biosynthesis and perception of the compounds can be accounted for by modulation of pre-existing systems.

Studies on similar species-complexes (*Ophrys fusca* s. lat. and *O. sphegodes* s. lat.) are presently underway. Both species-groups have a geographically disjunct distribution (allopatric). Furthermore, in both species-groups the geographical forms can be interpreted as separate species, which is a conclusion based on diverging flower morphology. The pollinator bee in all cases belongs to the same species. We hypothesize that these morpho-species have each independently acquired the same pollinator (PAULUS 1998, 2001), and thus their odor-bouquets must also be the same. The bee species responsible for pollination are *Andrena nigroaenea* and *Andrena flavipes*. The

males of *Andrena nigroaenea* pollinate *Ophrys fusca* s. str.³ (Mallorca), *O. sitiaca* (Crete) by means of abdominal pollination, and *Ophrys sphegodes* (southwest Germany, eastern Austria, southern Italian) as well as *O. cf. herae*⁴ (Crete) by head pollination. In contrast, *Andrena flavipes* performs pseudocopulation with allopatrically distributed forms: *Ophrys bilunulata* (Mallorca), *O. leucadica* (southern Greece, Rhodos, Samos, Chios, Kos), *O. funerea* (Sardinia), *O. africana – caesiella* (north Tunisia and Malta) and *O. israelitica* (Israel) (PAULUS & GACK 1990a, b, c; PAULUS 2001). New results indicate that all of these *Ophrys* species attract bees with the same odor bouquet of the so-called active compounds which are responsible for the specificity (STÖKL et al. 2005).

This would provide confirmation that the species-specific odor bouquet of other *Ophrys* species is created not merely by a few single ingredients but by a rich blend of components such as alkenes and alkanes. In particular, it appears that not just the type of volatile molecule is important but the actual mixture or proportion of alkanes and alkenes.

A completely different system of chemical mimicry is evident in the flower wasp *Dasyscolia (Camposcolia) ciliata* (Scoliidae) and *Ophrys vernixia* (= *speculum*). In light of current information, it operates with only a few different compounds of odor volatiles (ω -1)-hydroxy and (ω -1)-oxo acids, especially 9-hydroxydecanoic acid. The substances are very similar to those which attract the queen honeybee (AYASSE et al. 2003).

The question arises, what are the constraints which would induce *Ophrys* species in one instance to attract pollinator males with a very complex mixture and in other cases with just a few complex odor volatiles? The answer may lie in connection with the fact that *Ophrys* species-groups include many forms that are pollinated in turn by bee genera which themselves also contain many

³ *Ophrys fusca* s.str. is the typical, early blooming *O. fusca* Link described from Lissabon. DEVILLIERS & DEVILLERS-TERSCHUREN (1994) interpreted this species as belonging to the South-Spain *Ophrys colletes-fusca* (pollinated by *Colletes cunicularius*) and consequently they described the form pollinated by *Andrena nigroaenea* as a new species, *Ophrys lupercalis*. In spite of the pollinator of *Ophrys fusca* Link from Portugal is not known I think according to biometric analyses that *O. lupercalis* belongs to *O. fusca* (PAULUS 2001a). But the situation seems to be much more complicated as BERNARDOS ET AL. (2005) got different results. Further investigations are needed. Added July 2006: from my new investigations in Serra Arrabida in March 2006 *Ophrys lupercalis* is a species for its own.

⁴ The so-called *Ophrys herae* from Crete belongs to *Ophrys grammica* which is pollinated by *Andrena nigroaenea* (observations from Corfou, Levkas, Kephallonia and Crete. The true *O. herae* (from Samos) is pollinated by *Andrena thoracica*: HIRTH 2005, PAULUS unpubl., PAULUS & HIRTH, in prep.).

species. These *Ophrys* species should employ complex odor patterns to attract males, since the bee species themselves are genetically isolated from each other by recognition of complex blends. This would apply to bees from the genera *Andrena*, *Anthophora* and *Eucera* which pollinate *Ophrys* species. As shown in the table, a clear correlation is evident. An exception to this is the genus *Dasyscolia* (*Campsoscolia*) which contains only one European species. Similar reasoning applies to *Argogorytes* (Sphecidae), which is the pollinator of *Ophrys insectifera*. Although many components of the flower's scent are documented (BORG-KARLSON et al. 1987), the specific bouquet responsible for attraction has not been identified. It can be presumed that in this case the attractiveness is attained by just a few complex odor volatiles.

Another interesting question is whether virgin females exhibit the same attractiveness as the *Ophrys* flowers. To begin to answer this question, we performed an experiment toward the end of February, 1999, in Tunisia with *Ophrys vernixia* which showed that no essential differences in attraction existed. For this it was necessary to obtain females of *Dasyscolia* (*Campsoscolia*) *ciliata* which were not yet mated and to offer them in a choice-test along side flowers of *Ophrys vernixia* to the males. Since the visual stimulus of the flowers is more effective than that of the genuine females (see below), it was necessary to test each odor with identical optics. A virgin female was placed in a glass tube closed only at the bottom and a flower from *Ophrys vernixia* was inserted in second tube. Both tubes were outfitted with an *Ophrys* flower taken from the same inflorescence so that active males were presented on the right side with a flower plus female scent and on the left side with a flower plus flower scent. For the next 100 minutes, we counted the number of the approach flights that led to the male actually landing on the lip of the flower. Every 30 minutes the setup was moved about 10 meters to new position in another group of swarming males. The results were unambiguous. Both sides were chosen about equally, and one can conclude that under these experimental conditions the female scent is not more effective than the flower's.

2.1.2. Optical signals

In general, many male insects, and in our case, a male bee will rely on olfactory clues to follow the scent trail of a female, until he is able to visually perceive her. After that follow a chain of optical signals that function as releasing mechanisms which inform the males not only the exact location of the female but whether she belongs to the same species. As yet, no specific studies have been conducted to determine those features and colours used by males to recognize their females. Our information is limited to some very basic mechanisms that are inferred from numerous studies on shape recognition and

colour vision in the honeybee and a few select insects, and which most likely apply to further insects (CHITTKA et al. 2001, BRISCOE & CHITTKA 2001). To simplify the matter, we generally perceive our world as a flow of complex pictures, however an insect sees its visual world quite abstractly. In terms of behavioral biology, objects are the sum of their visual signals. They are evaluated by the receiver as “interesting” or “uninteresting,” as having the characteristics of a releasing mechanism or of something which must be remembered. Reactions to colour signs depend on motivation and the kinds of context. This is important for an interpretation of the appearance and colouration of *Ophrys* flowers with their high variable labellum patterns.

Even if there are hardly any systematic studies on the role optical signals play in *Ophrys* (KULLENBERG 1961, PAULUS 1988a), we may be permitted nevertheless to draw some general conclusions that are based on analogy and plausibility and that must await experimental verification.

Based on comparative studies of the pattern on the labellum of *Ophrys* flowers and on the strong assumption that they imitate the wings of a pollinator female with their shining surface, one can distinguish three categories of flower colouration and labellum pattern. The range of these categories is presumably an indication of what sorts of optical signals are important for males in recognizing their females in normal life. Underlying this hypothesis is the assumption that the pollinator selects those optical signals of the flower which, depending on his innate optical releasing mechanisms, serve as a minimum for female recognition. Since the optical recognition of females is relatively detailed, we can assume that the *Ophrys* flowers must imitate a large part of the visual signals to obtain approach flights and landings and to compete with other *Ophrys* plants of the same species in rate of visitation. In the context of mating behavior and sexual selection the males exhibit a form of male choice. Further underlying the hypothesis is also the assumption that evolution proceeds by opportunity and selection only as much as absolutely necessary. Selection is effective here as a form of intersexual selection since the pollinating males select among flowers of same species. The most female-like of the flowers achieve the highest rates of pollination (PAULUS 1988).

- The first category of flowers are those in which the optical signals play an important role in the sexual life of the pollinator male, so that the *Ophrys* flowers have been selected in this direction. Even for our eyes, the flowers reasonably resemble the females (colour fig. 1). The prime example of this flower category is *Ophrys vernixia* (= *speculum*) which, in a fitting way, is the same species that POUYANNE (1917) first discovered and recognized correctly a form of male deception (PAULUS 1978, PAULUS & GACK 1980). If one

compares the appearance of *Ophrys vernixia* flowers with the female of the pollinating species *Dasyscolia (Campsoscolia) ciliata*, one can easily recognize that the blue mirror of the labellum imitates the blue iridescence of the female's wings and the reddish brown hair-like structures on the labellum imitate the red body hairs of the female wasp. The sides of the labellum probably represent the middle and hind legs of the female. What other details are relevant here has not been examined. Conspicuous in many *Ophrys* species are the round and usually shiny basal calluses. They were incorrectly interpreted by DARWIN (1862) as false nectaries and later by many authors as eye-imitations. Actually, they are much more likely being imitations of the bare, scale-like structure over the base of the wings, the so-called tegulae. The reddish brown colour of the hairs of the labellum matches almost miraculously the colour of the body pubescence of the female wasp since this colour is only found in the west Mediterranean *Dasyscolia ciliata* ssp. *ciliata*. The east Mediterranean population of this species, *D. ciliata* ssp. *araratensis*, has a dark brownish red colour to the body pubescence. In accord with this, the colour of the labellum of the east Mediterranean *Ophrys vernixia* subsp. *orientalis*, is also darker (PAULUS 2001b)

- The second category of flowers is represented by those *Ophrys* species which to our eyes are clearly less similar to insects. They exhibit however patterns of colour and markings that only in retrospect after examining the female become understandable. Examples are *Ophrys cretica*, *O. ariadnae* and *O. kotschyi*. The black and white patches on their lip imitates the same colouration pattern on the females of the pollinator species belonging to the cleptoparasitic genus *Melecta (Melectini, Apidae)* (PAULUS & GACK 1983b, 1990a, b, PAULUS 1988b). The same applies to *Ophrys reinholdii* and *O. straussii* and its pollinator species, *Eupavlovskia* (PAULUS & GACK 1990a, b) which is currently regarded as a subgenus of *Melecta*. Some females of the mason bee *Chalicodoma (Megachilidae)* have a dark bluish hue and their wings a dark bluish iridescence. Some authors currently regard *Chalicodoma* as a subgenus of *Megachile*. The lips are correspondingly dark in *Ophrys atlantica*, *O. bertolonii*, *O. aurelia*, *O. benacensis* and *O. ferrum-equinum*. Furthermore, all of them have a dark blue iridescent spot on the top third of the lip. These *Ophrys* species are all pollinated by *Chalicodoma parietina*, the males of have a reddish brown colour (BÜEL 1978, PAULUS & GACK 1981, VÖTH 1984). Several *Ophrys* species (*O. spruneri*, *grigoriana*, *sipontensis*) also belong in this category and are pollinated by carpenter bees of the genus *Xylocopa (Xylocopinae)* which also have a blue-black iridescence (PAULUS & GACK 1990b, c; PAULUS 2003). At least with respect to *Xylocopa violacea*, we know that the blue-black shine of the wings is a visual element that is highly attractive to males (own unpubl. observations). Furthermore, the blue

iridescence is accentuated by ultraviolet light and probably also by polarized light. To add to this, the markings on the lips of *Ophrys* species always reflect in ultraviolet light (KULLENBERG 1961 and pers. data). This means that the reflection of marking on the lips represents an optical imitation of the wings. Ultraviolet light has a well-known high stimulation factor in a bee's life. Many plants have installed the use of ultraviolet light especially in the area of the pollen signs to build a contrast (OSCHE 1979, PAULUS 1988, LUNAU et al. 1996, LUNAU 2000). Also belonging to the second flower category are beautiful examples of convergences in basic colourations of the labellum. These *Ophrys* species are not closely related and have evolved similar or identical patterns on the lip of the flower in adaptation to the same pollinators. They include *Ophrys bertolonii*, *O. ferrum-equinum* and *O. atlantica* or *O. cretica*, *O. kotschyi* and *O. reinholdii* (PAULUS & GACK 1984, 1990, 1994).

- The third category of *Ophrys* flowers imitate only general colouration patterns of females belonging to the pollinator species. Most of the *Ophrys* representatives of this group are pollinated by bees of the genus *Andrena*, and include *Ophrys fusca*-aggr. and *O. sphegodes*-aggr. The lip of *O. sphegodes* (s. str.) matches the reddish brown or gray brown colour of the female in *Andrena nigroaenea* (PAULUS & GACK 1986, Paulus 1988b), while the very dark blackish brown of *O. mammosa* or *O. incubacea* match the dark body colour of *Andrena fuscata* or *A. morio* (PAULUS & GACK 1983b, 1986). The same applies to *O. spruneri*, *O. grigorigiana* or *O. sipontensis*, which are pollinated by bluish black species of *Xylocopa* (PAULUS & GACK 1990b,c). An agreement in basic colouration between the bee and orchid is usually found in comparison of closely related pairs of *Ophrys* species and in one case even within the same *Ophrys* species. An example for interspecific agreement among closely related pairs of species is *Ophrys omegaifera* (basic lip colour is maroon) and *O. fleischmannii* (dark olive brown). The females of pollinator species belong to the same subgenus of *Anthophora*, namely *Pyganthophora*: the reddish brown (as long as they are fresh) *Anthophora atrolba* is the pollinator of *Ophrys omegaifera*, and the gray olive brown *Anthophora sichelii* pollinates *Ophrys fleischmannii* (PAULUS & GACK 1983a, 1986). The example of intraspecific comparison has been previously mentioned and includes the allopatric subspecies of *Ophrys vernixia*, which diverge in colouration, and their common pollinator *Dasyscolia (Campsoscolia) ciliata*.

Other peculiarities of the lip drawings on *Ophrys* flowers are the so-called eyeglasses. They are found in the *Ophrys argolica*-aggr.: *Ophrys argolica*, *delphinensis*, *lucis*, *aegaea*, *biscutella* and *morisii* (possibly *O. climacis*, too). Also, there are the omega-markings on *Ophrys omegaifera*, *basilissa*, *dyris* and *O. fleischmannii*. All these species are pollinated by males of the genus

Anthophora. Experiments have not yet been conducted to unravel the meaning of the omega-marks. The high attractiveness of ultraviolet reflection from the lip-markings has been tested in experiments. For this purpose, two flowers were put inside a transparent box and *Ophrys* scent was blown from the outside next to the flowers with a Y-shaped tube. The scent of the flowers inside thus could not escape the container. In choice experiments the pair of flowers was presented to actively flying males. If males choose one flower significantly more often than the other, their choice must then be based optics. The front window of the box was made of normal Plexiglas or ultraviolet permeable plastic. Approaching males were thus given a choice between the two flowers and the significance of the ultraviolet reflection of the flower's lip could be tested.

Results from these choice experiments showed that the flowers which reflected ultraviolet light were more attractive than those that do not reflect ultraviolet light. Interesting is that result is obtained only in direct comparison. If males of *Eucera (Synhalonia) berlandi* (*Eucerini, Apidae*) are allowed to choose between two simultaneously offered scent-free flowers, they will only select a flower if one of them does not reflect ultraviolet light. In another context, the male will choose the flower which is unfamiliar to him. More on the learning experiments are mentioned later. We performed similar experiments with flowers of *Ophrys vernixia* and the pollinator males, *Dasyscolia (Campsoscolia) ciliata*, on the island of Mallorca. The results confirmed that flowers with ultraviolet reflection were much more often approached and landed on than flowers which had their ultraviolet reflecting areas blocked (PAULUS & ETTENAUER in prep.). KULLENBERG (1949, 1960) had already attempted to show in simple open-air experiments that the flowers function as a supernormal optical stimulus. In choice experiments he presented a female of the pollinator species alongside flowers either of *Ophrys lutea* or *Ophrys vernixia* (= *speculum*) to freely active males. The males unanimously chose the flowers as a copulation partner. Although the results sound enticing, they are nonetheless inconclusive, since the presented females were in all likelihood already mated. After a single mating females lose their attractiveness. The choice of the males is understandable because the flowers smelled like unmated females and were thus more attractive.

To compensate for this, we repeated the choice experiments with an apparatus that blocked out the undesired scents. Flowers of *Ophrys* and dead females of the pollinator species were offered without their respective scents to free flying males in choice tests. To accomplish this, flower scent was pumped through the Y-shaped tube so that it was emitted directly in front of each tested object, which, of course, was behind glass. Choice experiments with this setup were conducted on Crete with the species-pair *Ophrys heldreichii* and its pollinator

Tetralonia berlandi (Eucerini) [now named *Eucera (Synhalonia) berlandi*] (PAULUS 1988) and on Mallorca with *Ophrys vernixia* and *Dasyscolia (Campsoscolia) ciliata*. The results were in accord with those of KULLENBERG. The flowers were preferred more heavily than the females, yet now we can be sure that the males had chosen according to optical appearance. Based on numerous observations, in my opinion females of *Dasyscolia (Campsoscolia) ciliata* that have not yet mated, are able to advertise their readiness for copulation not only through their sexual pheromone but also by displaying their iridescence blue wings. They are spread flat over the body so they reflect with the greatest amount of surface. In contrast, females that have successfully mated hold their wings vertically against the sides of the body. At the same time they rhythmically raise and lower their wings, which inform the males that they have already mated. These females are, in fact, hardly ever bothered.

Based on these observations and on comparative studies of the markings on *Ophrys* flowers, it is safe to conclude that the flowers imitate the shine or reflective appearance on the wing of the female of the pollinator species. The wide variety of flower markings found in *Ophrys* species certainly corresponds to some extent with the range of optical cues that attract males. Of particular interest here are the highly complex markings on the flower lip of the *Ophrys holoserica-scolopax* group. These flowers are intricate with meandering and asymmetrical patterns. They vary from flower to flower to such an extent that no two flowers in a particular population may have identical patterns. From what we know up to now, these complex patterns are probably imitations of folded wings and may contain light reflecting patterns. Since they always reflect strongly in ultraviolet light, they should be easily perceptible by males. As will be explained in a latter section, experiments show that males are capable of memorizing individual patterns which they can recognize later, thus allowing them to avoid those females or flowers which were previously visited.

2.1.3. Tactile stimuli

After a male bee or wasp locates a female, it lands on her and begins with copulation attempts, several behavioral releasing mechanisms come into action. The male touches and probes the female with his body and the tarsi of his legs. By means of physical contact he registers the natural direction of her body hairs and possibly obtains additional chemosensory information. Based on the growth direction of the female's hairs, males decide immediately in which direction to position themselves. This is important especially when many males are scrambling for the same female. What matters is only who is the quickest and the first to insert their copulation apparatus. In and of it, the genital apparatus is a complex of important tactile sensory organs which no doubt gather further specific information. Unfortunately, there are no detailed examinations in this regard. However, flowers of *Ophrys* appear indeed to imitate some of the important tactile signals (AGREN et al.1984). For example, the growth direction of the hairs on the flower's lip. As KULLENBERG (1961) already showed all *Ophrys* species of the *O. fusci-lutea* group have a reversed direction of hairs on the labellum. All the fine glandular hairs run toward the stigma especially in the marginal areas of the tip of the labellum (colour fig. 2). In all other *Ophrys* species the hairs are directed from the stigma toward the tip of the labellum, and the flower markings are usually smooth. The effect achieved by these fine hairs is remarkable. The pollinator males instantly register the direction of the hairs after landing. In *Ophrys* species such as those belonging to the *fusci-lutea* group, the male immediately turns around and attempts copulation from the opposite position. This leads to removal of the pollinium with the tip of the abdomen (abdominal pollination). As previously mentioned, all other *Ophrys* species have hairs lying in the "normal" direction, so that the males remain in the forward position and remove the pollinium with the head (head pollination) (colour fig. 3). One of the first experimental analyses on hair direction and its tactile significance was conducted by PIRSTINGER (1996) on *Ophrys fusca* and *Ophrys sphegodes*. Both of these species are pollinated by the same bee, *Andrena nigroaenea*. In the first species this is accomplished by abdominal pollination and in the second by head pollination. Male bees can thus be found carrying pollinia in the front and in the back.

The size of the labellum is another very important aspect in this connection. It must roughly match the body size of the pollinator male. Males that are too large or too small are hardly capable of removing a pollinium and thus functioning as successful pollinators. It is possible to occasionally observe in the field that foreign bees or even other flower-visiting insects are not capable of assuming a proper position on the flower's lip. They usually prance about on the flower and fly off without having made contact with the sticky disc of the

pollinium. This results in selection for conformity in size between the length of the labellum and the body of the pollinator male. In fig. 1 are given correlations between body length of the pollinator male and its *Ophrys* species for 15 species pairs from head pollinations and 10 species pairs from abdominal pollination.

3. Pollinator specificity

Since each *Ophrys* species usually copies species-specific signals that trigger particular behaviors in the pollinator males (and only seldom in related species) the relationship between the plant and the male pollinator is necessarily highly derived. The mimicry functions best if the imitator is restricted to using a single species as its model. Imitating several females of various pollinator species would necessarily entail a greater compromise than imitating only one species. The highly integrated relationship is a natural consequence of selection for a particular pollinator species. In an *Ophrys* population which exhibits a certain variation in scent, only the males of a particular single species will ensure pollen transfer and thus also reproductive success. In having only one pollinator species, the *Ophrys* plant can capitalize on already well-functioning mechanisms that reproductively isolate the pollinator species from close relatives. The orchid, so to speak, parasitizes on the innate behavioral releasing mechanisms of the pollinator male. Pollinators thus represent for the *Ophrys* species a pre-gametic isolation mechanism which normally prevents hybridization through its high specificity (PAULUS & GACK 1983b, 1990c). In other words, it is a mechanism to ensure genetic or reproductive isolation that functions prior to pollination. Mechanisms of this kind have been called “prepollinating mechanisms” (LEVIN 1978, STEBBINS 1966).

Documentation of the highly specific relationship between *Ophrys* and its pollinator has been given for a series of species (KULLENBERG et al. 1984; PAULUS 1988b, 1997; PAULUS & GACK 1981, 1983a, b, 1986, 1990a, summaries in 1990b, c, d, 1994; VÖTH 1984, 1985, 1986, 1987; WARNCKE & KULLENBERG 1984). These studies verify the general thesis that for each species of *Ophrys* there is a single bee or wasp species responsible for achieving the pollination. In particular, our field observations and tests of the past 10 to 15 years in the entire Mediterranean area clearly show that this one-to-one relationship is nearly always present. We have not only attempted to identify the respective pollinator for each *Ophrys* species, but also whenever possible we have conducted choice experiments (biotests) under natural conditions. Males of a particular pollinator species were given opportunity to

freely choose between flowers of different *Ophrys* species. In nearly 100% of the cases, they correctly choose their own *Ophrys* species.

Pollinator specificity and the systematics of the genus *Ophrys*

Systematics and taxonomic ranks of taxa in botany are much confused because of lacking accepted criteria. Many botanists classify completely typological (anthropomorphic) without paying much attention to biological facts. The recent compendium of orchid names of KREUTZ (2004) is a deterrent example and offends all people attending for a scientific founded taxonomy.

Since the attraction of a pollinator to an *Ophrys* flower is essentially species-specific, the consequence is that pollen-transfer will be achieved only within a given *Ophrys* species. Accordingly, the *Ophrys* plants which are visited and pollinated by the same bee species are by definition to be regarded as species since the pollinator functions as a pre-pollinating isolation mechanism. Species specific attractions have been tested and confirmed in numerous choice tests in many regions of the Mediterranean (PAULUS & GACK 1990, 1995). Consequently, using the biotest as a criteria for ascertaining species status we could examine orchid populations and their respective flower morphologies as to whether or not they represent true species. Previously, about 25 to 30 species were recognized in the genus *Ophrys* (NELSON 1962, SUNDERMANN 1980, DAVIS & HUXLEY 1983, BAUMANN & KÜNKELE 1988, BUTTLER 1986). In the mean time, since many extraordinarily similar forms have become better known and can be separated, the number of valid species has risen to over 250 (DELFORGE 2005). At present, an end to this trend is not foreseeable, because some areas, especially the eastern Mediterranean, have not yet been adequately studied.

From these results, it follows that the process of species formation in the genus *Ophrys* always proceeds with the acquisition of a new species of pollinating males. In the ensuing evolution the males breed, so to say, "their" flowers to accord with their image of females. This selection leads to differences in the morphology of flowers of *Ophrys* plants that legitimize us to regard the different forms as distinct species, in as much as species can be defined according to morphological criteria and despite not yet knowing the pollinator species. Verification of such a preliminary assessment on the validity of the status of an *Ophrys* species can be obtained by subjecting the pollinator to a biotest. In addition, we must not forget that pollinator species have selected not just for flowers according to morphology but, and even more so, for their scent production. Molecular analyses are in progress. Our first results demonstrate that even very near related *Ophrys* species (e.g. species of the *O. fusca* group) could separated only by AFLP-techniques because the common nuclear

ribosomal ITS sequence investigations are not able to make sufficient resolutions within the genus (SCHLÜTER et al. 2005a, b). This is strongly evident in the new publication on Iberian *fusca*'s (BERNARDOS et al. 2005).

Two main processes of species formation can be formulated which apply to *Ophrys* and its pollinators – adaptive radiation, allopatric and possibly sympatric species formations. Adaptive radiation leads to groups of *Ophrys* species that have acquired pollinators which are likewise closely related. Switching to a new pollinator in this situation is easily conceivable since closely related bees probably also possess a similar scent production. In this way, *Ophrys* species-groups evolved in conjunction with pollinator species that are in particular from closely related bees in large genera such as *Andrena* and *Eucera*. Switching to a new genus of pollinators may occur with the formation of new *Ophrys* species which exclusively adapt to their new pollinators. Thus, males of various species of *Anthophora* pollinate members of the *Ophrys argolica* group by head pollination and they also pollinate the *Ophrys omegaifera* group by abdominal pollination (table 2).

There is however evidence for the formation of species accompanied by a huge change of pollinator species. One species from southern Spain, which has not yet been named, is pollinated by the bee *Colletes cunicularius* (Colletidae) (PAULUS & GACK 1981). A second closely related species, *Ophrys atlantica*, also in southern Spain, is pollinated by the mason bee *Chalicodoma parietina* (Megachilidae) (PAULUS & GACK 1983). A third relative, *Ophrys blitopertha*, in the eastern Aegean, is pollinated by the scarab beetle *Blitopertha lineolata* (Scarabaeidae) (PAULUS & GACK 1990a, Paulus 1998). This represents an immense jump in pollinator acquisition. Perhaps, even more fascinating is the trend in pollinator acquisition in the *Ophrys insectifera* group. The Central European fly-orchid, contrary to its common name, is pollinated by a species of digger wasps, *Argogorytes mystaceus* (Sphecidae) (WOLF 1950, KULLENBERG 1960) and sometimes from the near related *A. fargei*. However, the closely related *Ophrys aymoninii* in southern France has the bee *Andrena combinata* as pollinator (PAULUS & GACK 1990a). Another close relative, *Ophrys subinsectifera*, has recently been described from northern Spain. It has acquired two pollinator species which belong to the sawfly family *Argidae* (Hymenoptera): *Sterictophila gastrica* and *S. furcata* (HERMOSILLA et al. 1999).

Table 2

List of *Ophrys* species belonging to two species-groups that are pollinated by male bees of *Anthophora* (= *A.*) (Anthophorini, Apidae).

Ophrys	Pollinator	Locality	Literature
<i>argolica</i> group (head pollination)			
elegans	<i>A. erschowi</i>	Cyprus	PAULUS & GACK (1990b)
aegaea	<i>A. orientalis</i>	Karpathos	PAULUS & GACK (1990c)
lucis	<i>A. cf. mucida</i> = <i>biciliata</i> (?)	Rhodes	PAULUS & GACK (1990c)
argolica	<i>A. plagiata</i>	southern Greece	PAULUS & GACK (1990a,c)
delphinensis	<i>A. plagiata</i>	southern Greece	PAULUS & GACK (1990a)
biscutella	<i>A. retusa</i>	southern Italy	PAULUS & GACK (1986)
morisii	<i>A. sichelii</i>	Sardinia, Corsica	PAULUS & GACK (1995)
crabronifera	<i>A. plumipes</i>	western Italy	PAULUS & GACK (1995)
provincialis	<i>A. atriceps</i>	southeastern France	PAULUS & GACK (1995)
<i>omegaifera</i> group (abdominal pollination)			
omegaifera	<i>A. dalmatica</i> ⁵	Crete, Karpathos, Rhodes, Naxos	PAULUS & GACK 1986
	<i>A. nigriceps</i>	Rhodes, Samos, Chios	unpubl.
basilissa	<i>A. nigrocincta</i>	Crete, Kos, Samos	PAULUS 1988 & unpubl.
fleischmannii	<i>A. sichelii</i>	Crete	PAULUS & GACK 1986
dyris	<i>A. atroalba</i>	southern Spain	PAULUS & GACK 1983
dyris (f. "balearica") ⁶	<i>A. balearica</i> ⁷	Mallorca	PAULUS & AYASSE (in press)

We possess no information on the odor bouquet of these species, and I cannot explain in detail how *Ophrys* pollination can jump between bees, digger wasps and sawflies.

⁵ Formerly known as *A. atroalba* ssp. *agamoides*

⁶ Closely related to *dyris*, considered mostly as conspecific.

⁷ Possibly a subspecies of *atroalba*

4. Is there pollinator selection on *Ophrys* flowers?

4.1. Indirect evidence from comparative investigations

The exact agreement between the odor bouquet of the *Ophrys* flower and the scent of the females of the pollinator species suggests to us that it has resulted from selection pressure exercised by the males. They bestow those flowers a high reproductive success that best correspond to their expectations of a female. Flowers with a less attractive or unattractive scent are much less likely to be pollinated. As mentioned previously, comparative studies have shown that *Ophrys* species with a disjunct geographical distribution employ the identical pollinator with identically composed odor bouquets. Up to now, species from the *Ophrys fusca* group and from the *Ophrys sphegodes* group have been investigated (STÖCKL et al. in press). This should, of course, also apply to optical signals. Visual signals are more readily understood since we are able to make direct comparisons. Excellent examples of this are *Ophrys bertolonii* (Italy), *O. ferrum-equinum* (Greece) and *O. atlantica* (southern Spain and northern Africa). All these *Ophrys* species are pollinated by the same species of mason bee *Chalicodoma parietina* (Megachilidae). Since the three *Ophrys* species are not closely related to each other, their common optical signals must have developed convergent. When a convergence, i.e., an independent acquisition of optical signals, is evident within a closely related group of *Ophrys* species, we speak of parallel evolution or parallelisms. The following species-pairs provide examples of parallelisms: *Ophrys apulica* (southern Italy) - *O. heldreichii* (southern Greece) - *O. episcopalis* (southern Aegean) or *Ophrys kotschyi* (Cyprus) - *O. cretica* (Crete, Rhodes) (PAULUS & GACK 1986, PAULUS 1988). However, we rapidly encroach upon the area in evolutionary systematics where it is no longer possible to distinguish between relatedness and convergence. To avoid this dead end, studies have begun which rely on the methods of molecular systematics (BATEMAN et al. 1997, PRIDGEON et al. 1997, ACETO et al. 1999, SOLIVA et al. 2001). The results so far, however, have contributed little to our understanding of *Ophrys* at the species level.

4.2. Direct evidence, experiments on pollination success rates

4.2.1. Height of stem and male-patrolling altitude

In order to postulate that pollinators exercise selection on the flowers, it must be shown that males influence the reproductive rate of the orchids they choose. This presupposes that the flowers within a population are variable and that the males react with different frequencies of approach flights and landings (PAULUS 1988). A series of field experiments dealing with this has already been conducted. PAULUS & GACK (1980) were able to show that the flower

height of *Ophrys vernixia* is a relevant factor. Actively flying pollinator males of the scoliid wasp *Dasyscolia ciliata* were presented flowers at heights of 5 cm, 10 cm and 15 cm above the ground. The numbers of landings were registered over a period of 150 minutes in 30 minute intervals (fig. 2). The results were astoundingly clear. The lower the height of the flower, the more frequently it was chosen. This agrees with observations that males search for females by patrolling just above the ground level. In fact, *Ophrys vernixia* belongs to those species that are characterized by a rather low lying inflorescence. This agreement between low plant growth and low male search flight does not apply to other *Ophrys* species, since males from other pollinating species generally patrol around bushes and over well-grown fields, etc. A negative selection pressure could take place in some orchids if they have high growth form. However, the *Ophrys* species which are pollinated by species of *Anthophora* or *Eucera*, can attain considerable growth heights.

4.2.2. How often do males visit *Ophrys* flowers?

It has been noticed on many occasions that although males are intensely attracted to the flowers, their interest rapidly recedes after a short period (PAULUS et al. 1983). In order to investigate this more closely, we conducted a series of learning experiments. Two hypotheses were postulated:

1. After visitation and especially after pollination flowers quickly become unattractive, and they have halted odor-production.
2. The males learn to recognize individual flowers in order to avoid repeated visitations. The learning of individual flowers presupposes that flowers are individually different (PAULUS 1988).

Learning experiments were performed in southern Spain, Crete and Tunisia. After the *Ophrys* species and its species-specific pollinator were found, we searched for areas where plenty of males were swarming. In these areas we placed potted *Ophrys* plants that were in bloom. After completion of the experimental series all plants were returned to their original sites. The following pairs of orchids and bees were examined in this respect:

<i>Ophrys heldreichii</i>	<i>Eucera berlandi</i>	Crete (Greece)
<i>Ophrys picta</i> (<i>scolopax</i> pars)	<i>Eucera barbiventris</i>	southern Spain (Malaga)
<i>Ophrys tenthredinifera</i>	<i>Eucera nigrilabris</i>	northern Tunisia

1. One potted plant each of *Ophrys picta* (= *scolopax* pars), *Ophrys heldreichii* and *Ophrys tenthredinifera* were placed in the male swarm area and the rate of landings was measured. Previously, we determined through observation, capture, marking and releasing of males that the area constantly contained about 10 to 12 males. This number agrees with the

older observations of HAAS (1960). The results were always identical. At first, and very quickly, nearly all of the males in the swarm attempted to copulate with the various flowers of the plant. Within 15 to 30 minutes their interest had waned so that no further landings were registered. We repositioned the orchids several times within the swarm areas to discount spatial learning (fig. 3).

2. Afterwards the orchid plant which was originally presented was replaced with a new specimen. Immediately the rate of approaches and landings dramatically increased and after a short period fell to zero again. This procedure can be repeated two or at most three times before the males completely lost interest (fig. 4).
3. The plants which were tested in one area were brought to a nearby second swarm area in which different males flew. These males were thus naive to *Ophrys* flowers. The behavior of these males was essentially the same as the previous experiment. Almost instantly, the majority of males came to perform copulations with the flowers. Their interest quickly resided and could only be awakened again when the presented plant was replaced with one that they had not already experienced.
4. When the males were offered a plant that had already been presented to them, the rate of landing did not increase (fig. 4).

From these results the following conclusions can be drawn:

1. The attractiveness of the flowers does not decline simply by the fact that they have been previously visited because the flowers release immediate copulation attempts when presented to new and inexperienced males.
2. One possibility is that the males mark visited flowers with a scent (anti-aphrodisiac) (KUKUK 1985). To exclude the possibility that males may mark with a personal individual odor, we presented actively flying males with flowers that were sliced lengthwise in half. When the rate of landing reached zero, the flowers were exchanged by their respective halves. In all cases, the rate of landing did not increase. When males are subsequently offered a flower-half from a different plant individual, it was immediately chosen.
3. It is thus apparent that male bees possess the ability to remember individual flowers in order to avoid them later. Accordingly, each and every plant must differ individually and these differences must be recognizable to the males (PAULUS et al. 1983, PAULUS 1988).

4.2.3. Pollinator males can distinguish different flowers from different plant individuals

Visual signals, as well as olfactory signals, are distinctive and therefore provide a basis for learning. It is easy to recognize that the labellum pattern in species of the *Ophrys holoserica-oestrifera* group is exceedingly variable and complex – hardly any individual plants have identical patterns. The flowers of an inflorescence however have virtually identical patterns. To examine male memory we devised two series of field experiments to test the positive differentiation of two flowers simultaneously presented. If males are able to distinguish between them, and if we assume it is of value to them, then the landing rate should reflect clear differences between the flowers. A differential preference by males may be based on the nearly identical lip pattern on the flowers, and probably as well also the nearly identical odor-bouquet.

1. Two flowers from the same plant individual: The expected result is that they will not be distinguished from each other and, if so, then only somewhat. For this test, two flowers at a time were laid on a neutrally coloured background and presented to actively flying males. The flowers were exchanged left and right every minute to exclude the possibility of preference for one side or the other. The number of landings on each flower was recorded.
2. Two flowers from different plant individuals: One can expect here that they will be distinguishable from each other and that the landing rate will differ significantly.

These kinds of experiments confirm the anticipated experimental results. Males were much less able to differentiate two flowers from the same inflorescence, with a ratio of 1 to 2, than two flowers from two different plant individuals, ratio of 1 to 9. It is remarkable that flowers on the same inflorescence can be distinguished although their patterns are the same.

Since this experiment could not determine whether the males use visual or olfactory cues, another series of experiments was performed in which the males had to rely only on visual signals. The flowers were placed in clear plastic boxes, the sides of which were permeable to ultraviolet light. In this manner the scent of the flower was not able to disperse. Since males generally do not respond to optical signals of *Ophrys* alone, flower-scent was pumped to the outside of the boxes with a Y-shaped tube. Thus each box had an identical scent and male choice could be based only on optical stimulation. Since the males were prevented from actually landing on the flower, we counted as a positive reaction what appeared to be a clear attempt to land.

- A. Two flowers from the same plant individual: The expectation is that they will not be distinguished from each other since they are visually identical.

B. Two flowers from two different plant individuals: We should expect here roughly the same difference as in the open flower experiment.

The results of these tests were similar to those before. The two flowers from different plants were clearly chosen at different rates. The two flowers from the same plant individual were, in this case, not distinguished at all. Two conclusions which can be drawn from these results.

1. Plants of the same species (in our case, *Ophrys heldreichii*) exhibit individual variation within the limits of its species specificity. In addition to a highly variable visual pattern on the labellum, there must also be an individuality of scent.
2. Individuality of scent is even apparent on different flowers of an inflorescence.

Analyses on the chemical components of the scent of *Ophrys sphegodes* verify the conclusions reached by experimental analysis. The studies by Ayasse et al. (1997, 2000), SCHIESTL et al. (1997a,b; 2000) and, in particular, SCHIESTL & AYASSE (2001) demonstrated the existence of a species-specific scent, in addition to an individual scent for individual plants and even for individual flowers of same inflorescence. The results were confirmed in various biotests performed on the males of *Andrena nigroaenea*.

5. Optimal pollination and avoidance of self-fertilization

Let us briefly recapitulate the significance of the results, observations and experiments mentioned so far.

- Males are attracted to *Ophrys* flowers which produce in a species-specific manner exact copies of the female sexual attractant (SCHIESTL et al. 1999, 2000; AYASSE et al. 2003). They attempt to mate with the flower in approximately the same manner as they would a genuine female.
- After several further unsuccessful mating attempts the males learn to avoid that particular flower. The flower is treated in a manner as if it were an unattractive female (PAULUS et al. 1983, PAULUS 1988). Learning or habituation in this fashion also occur in male bees encountering genuine females that are not willing to accept these males (unattractive) (SMITH 1983).
- Bee females, as well as the *Ophrys* flowers, emit a species-specific scent plus additional individual scent-compositions, which provide a basis for learning (PAULUS 1988, AYASSE et al. 2000).
- An individual recognition of female bees is beneficial to avoid mating among siblings. When males can choose between closely related females or

less closely related females, they select those of a more distant relationship (BUCKLE & GREENBERG 1981, HEFETZ et al. 1986, BARROWS et al. 1975, MICHENER & SMITH 1987, SMITH & AYASSE 1987, WCISLO 1992, AYASSE et al. 2001).

This type of individual variation in olfactory compounds and in colour signals can only be explained in connection with optimizing the rate of pollination, since differences in landing rates lead to differences in reproductive success (PAULUS 1988, 1997; AYASSE et al. 2000). It must also be recognized that, in general, orchids are prone to greater problems with self-pollination than other flowering plants. The invention of pollinia was a necessary solution to ensure that with only few visitations, large amounts of pollen can be transferred to the stigma. In this manner, each flower produces an immense amount of seeds. The number of seeds in orchid flowers ranges between 15,000 and one million (NAZAROV & GERLACH 1997 and many own countings). Even self-fertilization in this kind of pollination-system may entail fatal consequences, since large amounts of pollen may be incorrectly transferred or lost. Further special adaptations are necessary in order to avoid self-pollination. One of these adaptations is the development of deceptive methods which, although they promote the attraction of a potential pollinator, they also discourage repeated visitations from the same pollinator individual by providing no rewards or even by “frustrating” the visitor. This is the “pollinium hypothesis” or “self-pollination avoidance hypothesis” proposed by PAULUS & GACK (1980, 1990), PAULUS (1988, 2005), and reviewed by NILSSON (1992a). Since the pollinator males possess learning capacities, they avoid these flowers in the future. This applies also to the so-called food-deception flowers (*Orchis*, *Dactylorhiza*, *Cephalanthera*, etc.) (e.g. GUMPERT & KUNZE 2001), and to the instinct-deception flowers of *Ophrys*, which as already mentioned provide individual odor-bouquets to assist in the learning and recognition of individual flowers. The colour-polymorphisms of *Dactylorhiza sambucina* and *D. romana* can also be interpreted in this light (NILSSON 1980, PETERSON & NILSSON 1983, LAGUTOVA & NAZAROV 1991, GIGORD & AL. 2001). The pollinium hypothesis adequately explains the fact that especially Orchidaceae have evolved a wide variety and amount of pollination systems based on deception. Pollination by deception is found in over 70% of all orchid species in the European flora. The only other plant group which also has developed pollinia is the *Asclepiadiaceae* (milk weeds). Presumably for the same reasons, they have also developed a high number of deceptive flowers (OLLERTON & LIEDE 1997). To maximize pollination, *Ophrys* flowers have developed further tricks. As already indicated in choice-experiments with pairs of *Ophrys* flowers (PAULUS 1988, PAULUS & GACK 1994), the odor-bouquets are not identical. The gas chromatographic analyses show that differences in odor are based on different

ratios of alkanes and alkenes in the lip of the flower (SCHIESTL et al. 1997b, AYASSE et al. 2000). Because of the avoidance reaction in males that have been deceived before, they will fly with a higher probability to the next flower of the same inflorescence. In this manner, a second pollinium can be removed and if the bee is already carrying a ripened pollinium from a previous visitation, it will pollinate the second flower. The previously removed pollinia are capable of pollination only after two to three minutes, after the stalk has dried and bend forward.

Ophrys flowers have developed clever mechanisms which cause males to regard those flowers that have already been pollinated as unattractive. Since a single pollination event will transfer more pollen than is necessary for the number of ovules (PAULUS 1988), an *Ophrys* flower obtains no benefit from additional visitations or pollination attempts. Anything more than a single pollination event means wasted pollen. The male's initial interest in the flower should be redirected to other flowers of the same inflorescence which have not yet been visited. This can be achieved in at least two conceivable manners. Since after mating, females halt production of the sexual attractant or even begin production of an anti-aphrodisiac (SCHIESTL & AYASSE 2000), the *Ophrys* flowers, too, may respond in a similar fashion. Laboratory and field experiments with *Andrena nigroaenea* and *Ophrys sphegodes* (Vienna), as well as *Eucera nigrilabris* and *Ophrys tenthredinifera* (Tunisia) clearly show that males can differentiate between a pollinated flower and an unpollinated flower. In acceptance-tests, we placed males of *Andrena nigroaenea* on flowers of *Ophrys sphegodes* and observed the extent of their sexual excitement. A positive reaction was registered if they showed vehement wing-buzzing in combination with copulatory movements (reaction type 1), just a short wing-buzzing (type 2), however, merely walking about and then flying off, was counted as a negative reaction (type 3). Each male was placed on a pollinated and then an unpollinated flower for a total of ten alternations. In an additional series of these types of tests, the males were tested first with cross-pollinated flowers (allogamy) and then with self-pollinated flowers (autogamy). The results of the three types of reactions are summarized in fig. 5. As anticipated, the males exhibited nearly always rigorous reactions on the unpollinated flowers and only rarely on the cross-pollinated flowers. It is noteworthy however that some males showed copulation behavior on self-pollinated flowers, even if to a much lesser extent than on unpollinated flowers. In field tests with males of *Eucera nigrilabris* in Tunisia, I obtained entirely comparable results. A chemical analysis of pollinated and unpollinated flowers of *Ophrys sphegodes* along with complementary biotests in the field with males of *Andrena nigroaenea* demonstrate that male rejection of pollinated flowers is based on the production in successfully pollinated flowers of an anti-aphrodisiac, farnesylhexoanate, which is the same substance produced by the

mated female bees (SCHIESTL et al. 1997a, SCHIESTL & AYASSE 2000). A different case is found in *Ophrys vernixia* and the males of *Dasyscolia* (*Campsoscolia*) *ciliata*. It must be remembered that the females produce a sexual pheromone that consists of few different types of odor volatiles belonging to the group of hydroxy acids and that this substance is also produced by the flowers. After successful mating, or after successful pollination, its production is reduced in the female as well as in the flower (AYASSE et al. 2003).

Speciation

All species of *Ophrys* examined, so far, possess a single pollinator which is specifically lured. This has been confirmed by field observations, numerous biotests and above all chemical analysis of scents. This is an evolutionary product of an equally specific selection by the pollinating males to benefit only those plant individuals which come closest to their expectation of a female. Further evolutionary trends concern flower characteristics and flower morphology, i.e., characteristics which are important in taxonomic decisions. The males function as a very effective pre-mating isolation mechanism, or to put it more aptly, as a pre-pollination isolation mechanism for the *Ophrys* species they pollinate. *Ophrys* species can thus be defined as biological or evolutionary species. This means that the formation of each new species must be accompanied by acquisition of a new pollinator. Allopatric speciation in the genus *Ophrys* is easily conceivable as it is in many other animals and plants. It requires demonstration of historical separation of geographical areas, splitting and reunion of islands, raise and fall of sea levels, appearance of land bridges during the ice periods, etc. That sympatric speciation also has played a role is indeed conceivable, but difficult to demonstrate. Sympatric speciation would mean that a part of the population, despite continuous contact with the remaining, could acquire a different pollinator and prevent back-crossing. With regard to the closely related species of the *Ophrys fusca* group, we know that their order bouquets contain more or less the same alkanes and alkenes, and that the specificity of the odor results from a special blend of these volatile compounds (SCHIESTL & AYASSE 2002). Species formation and thus acquisition of a new pollinator can be brought about by a shift in the relative amounts of the components of the blend. This evolutionary pathway has probably been taken very often to judge by the amount of natural variation in a population. The following is a plausible scenario for species formation. Consider a hypothetical species of *Ophrys*, and that it flowers over a period of four to six weeks. At the beginning of the blossom period, it is frequented by a different species of *Andrena* as toward the end of this period. When the flight activity of the males of both species are sufficiently separate, then the

development of two species of *Ophrys* can proceed, which will have staggered periods of blossom. Actually there are many examples of closely related flowers of the *Ophrys fusca* group which have staggered periods of flowering (PAULUS 1998, 2001). These are examples of niche formation created by blossom periods which do not significantly overlap.

The formation of new species by introgression (homoploid hybridization) is regarded as a common method of speciation in botany. An even more widely occurring form of speciation among plants is assumed to be hybridogenetic polyploidy and allopolyploidy (ANDERSON 1953, EHRENDORFER 1959, 1980). However since the chromosome number is identical in nearly all species of the genus *Ophrys*, this method is not probable. The formation of new species by hybridization or introgression has been previously assumed to occur for the species of the genus *Ophrys* (STEBBINS & FERLAN 1956, EHRENDORFER 1984, DANESCH et al. 1975). This remains the most likely possibility, yet no verifying evidence so far has been brought forward. PAULUS & GACK (1995) and PAULUS (1997) described the case of *Ophrys chestermanii* on Sardinia and its alleged hybrid with *Ophrys tenthredinifera*: *Ophrys normanii*. It is remarkable that the sympatrically occurring *O. chestermanii*, which belongs to the *O. holoserica* group, and *O. normanii*, which belongs to the *O. tenthredinifera* group, both have the same pollinator, the cuckoo bumblebee *Bombus (Psithyrus) vestalis*. Preliminary chemical analysis of the odor-bouquet showed that *O. normanii* possesses numerous substances that are not found in the alleged parental forms. Future studies should employ population genetic methods to evaluate the presumed introgression despite the genetic influence of identical pollinators.

Origin of sexual deception

Sexual deception presumes that flowers invent signals which primarily lure only males and preferably only those from one or a few closely related species. One general pre-adaptation for this is that the use of scent is widespread among flowering plants. This means that the flowers only have to concoct the right mixture. The first hypothesis formulated in this direction was proposed by VOGEL (1972). He observed on the island of Elba that males of the bee species *Eucera nigrescens*, while patrolling their territorial swarm areas, may be applying a scent marking to the flowers of *Orchis papilionacea*. If these flowers were capable of producing the scent mark themselves, it would attract swarming-marking males that could ultimately lead to pollination. Over the years we have attempted to find evidence for a close connection between species of *Eucera* and species of *Orchis*, which would be indicative of scent

marking as suggested by Vogel, but without any success. In short, the hypothesis could not be confirmed. Although this kind of scent imitation would guarantee attraction of males only, it does not represent an essential step toward releasing copulation behavior. The fact that pollination by means of sexual deception has originated on multiple occasions worldwide only in orchids shows that this family obviously possesses excellent prerequisites. One of these is described in the pollinia-hypothesis. Pollen transfer is achieved by pollinia which are packets of enormous amounts of pollen that ensure an exceptionally high rate of pollination. Crucial is that only two visitations are statistically required for complete pollination: one for the removal of a pollinium and one for pollination on a second flower. The amount of pollen which is transferred suffices for fertilization of all ovules (PAULUS 1988). This opens up the possibility for developing unusual mechanisms of attraction which enable the animals carrying pollinia to learn not to return to the same flower. This serves to minimize self-pollination. Two main methods of deceptive attraction are common in the *Orchidaceae* – food deception and sexual deception. With regard to the European flora, over 70% of the species follow one of these strategies. This is ample implication of how successful pollination by deception is. The Australian flora includes about 230 species of orchids contained in eleven genera which practice sexual deception. In South Africa two species of *Disa* must be mentioned in this context (STEINER et al. 1994, JOHNSON et al. 1998). To what extent this phenomenon is dispersed in southern Asia and South America is poorly known (VAN DER PIJL & DODSON 1966, DAFNI 1984, SINGER 2002, SINGER et al. 2004, BLANCO & BARBOZA 2005). Interesting is that in the Australian genus *Caladenia*, there are species which rely on pseudocopulation and others which depend on food-imitation. This example shows that even in Australia sexual deception must have evolved on several independent occasions. It also reveals that the evolutionary pathway started with food-imitation. We do not know what pollination-system was present in the forerunner to the genus *Ophrys*. The search for the phylogenetic sister group to *Ophrys* has not yielded conclusive results. According to the DNA sequence analysis of ACETO et al. (1999), the clade *Barlia* + *Himantoglossum* is the sister group to *Ophrys*. According to PRIDGEON et al. (1997) it is the clade *Orchis* + *Anacamptis* + *Serapias*. And finally, according to the study of SOLIVA et al. (2001) the sister group is *Serapias* alone (fig. 6). In any case, it is safe to conclude that *Ophrys* is probably derived from a clade of food-imitating flowers. With this in mind, DAFNI (1987) following the work of BINO et al. (1982) proposed a model in which the only the males of *Lasioglossum marginatum* (*Halictidae*) visit the food-deceiving flower *Orchis galilaea*. He interpreted the behavior of the males to be at least partly guided by the presence of sexual olfactory cues. However, the males apparently search only for nourishment on the pollen-markings of the lip of the *Orchis* flower. A

similar case is found in the relationship between *Chelostoma uliginosum* (*Megachilidae*) and *Cephalanthera rubra*. NILSSON (1983) was able to show that the orchid flowers imitate important visual and probably also olfactory signals of *Campanula* species. It must be mentioned that the males normally search for females while they are foraging inside flowers. According to my own investigations, the orchid flowers act similar to *Orchis galilaea* in that they attract only those males that are searching for nourishment (PAULUS unpubl.). However going beyond these observations, it may be possible that sexual deception occurs, since the orchid flowers may be imitating not just a *Campanula* flower, but a *Campanula* flower with a receptive female sitting inside. In any case, that would be the working hypothesis.

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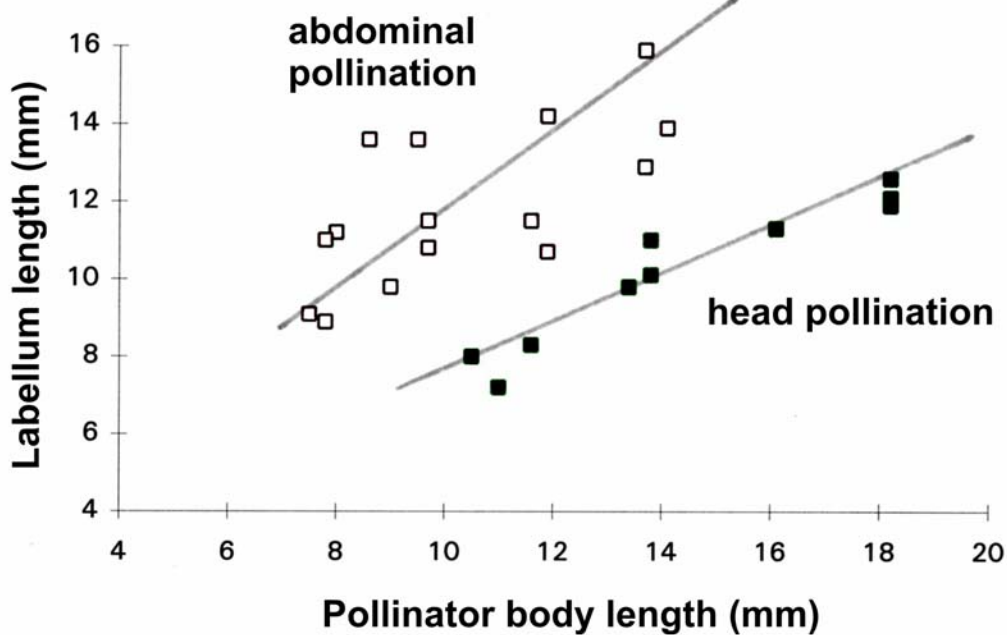


Fig. 1

Correlation between labellum length of 25 *Ophrys* species with body length of the pollinator male species. Each dot represents the pair of *Ophrys* species with its pollinator species. Each dot is the median value of 10 individuals.

There are correlations between pollinator's body length and labellum length of the respective *Ophrys* species: for the head pollinated *Ophrys* species this correlation is strong, for those of the abdominal pollinated species it is good. In cases of abdominal pollination it is less important how long the part of the labellum is in front of a pseudocopulating male. For head pollinated *Ophrys* species it is important that the male fits exactly on the whole labellum; because only in this case the pollinaria can be removed.

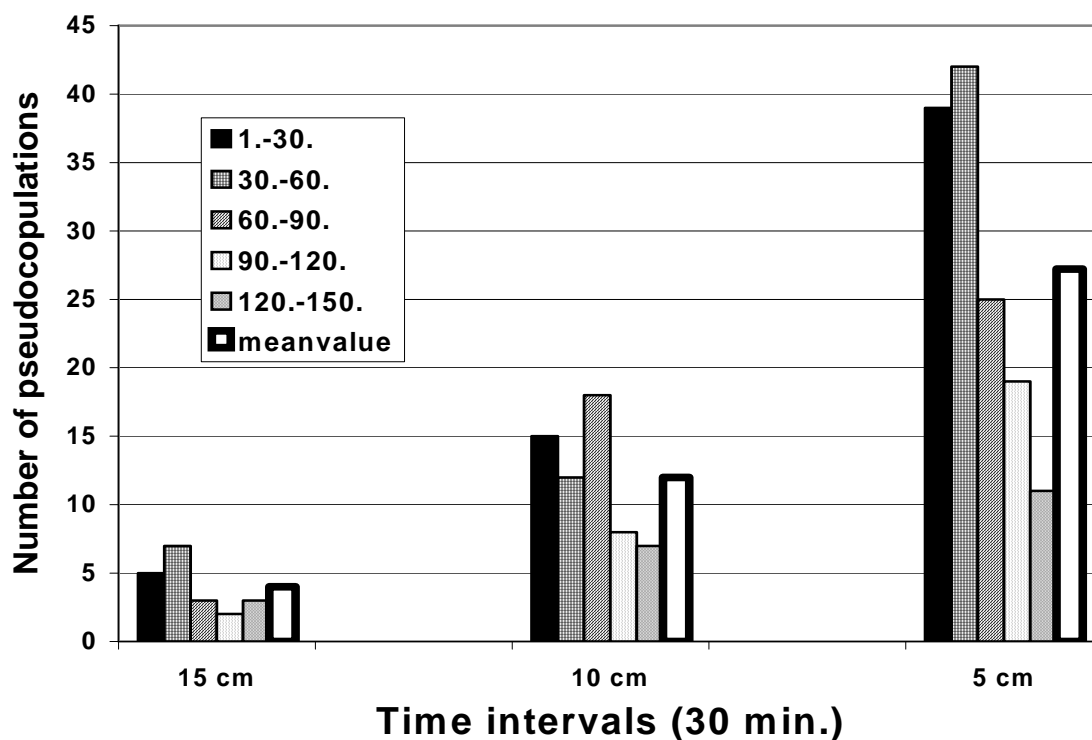


Fig. 2

Number of pseudocopulations of *Dasyscolia ciliata* with flowers of *Ophrys vernixia* depending on growth height above ground. Three flowers were presented in patroulling areas of males of the scoliid wasp: one at 15 cm, one at 10 cm and the third in 5 cm above ground. During 150 minutes all pseudocopulations were counted summed up in 30 minutes intervalls. The most attractive flower was this at 5 cm heigh above ground level. That means that the pollination success in *Ophrys vernixia* is depending on the growth heigh of flowers (redrawn and changed after PAULUS & GACK 1980).

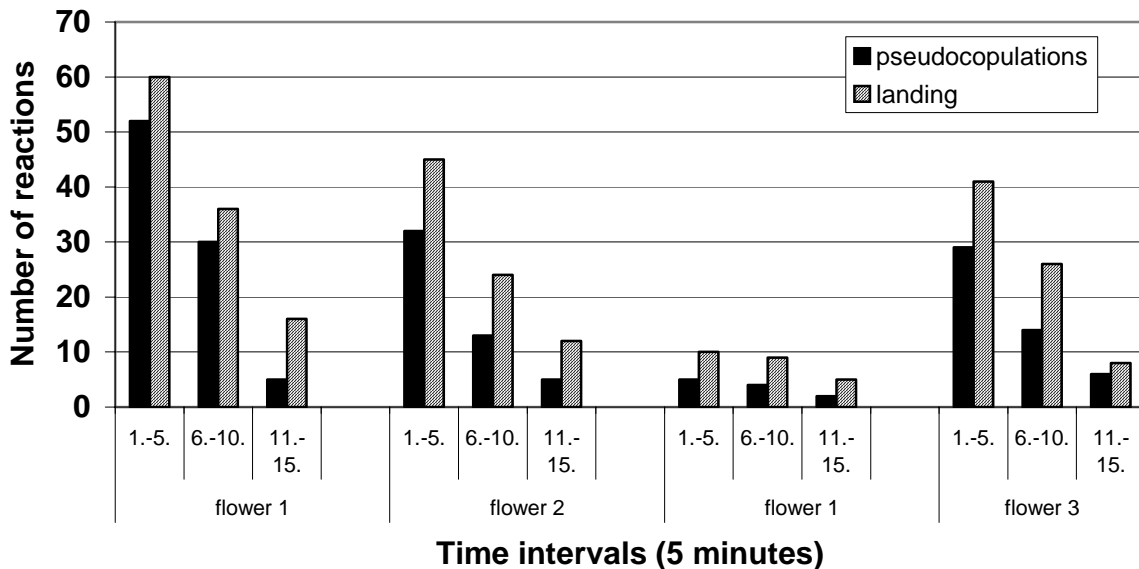


Fig. 4: Number of pseudocopulations on 3 flowers of two different plant individuals of *Ophrys tenthredinifera* with *Eucera nigrilabris*. To demonstrate that the males learn the flower individually the first flower was presented after 45 minutes again. To show that the low landing rates on flower 1 was not a consequence of lacking motivation, a third new flower was presented. Low numbers of landings demonstrate that the bees had learned the first flower. Observations in Tunisia, March 1999, sum of 3 experiments, n=489 landings.

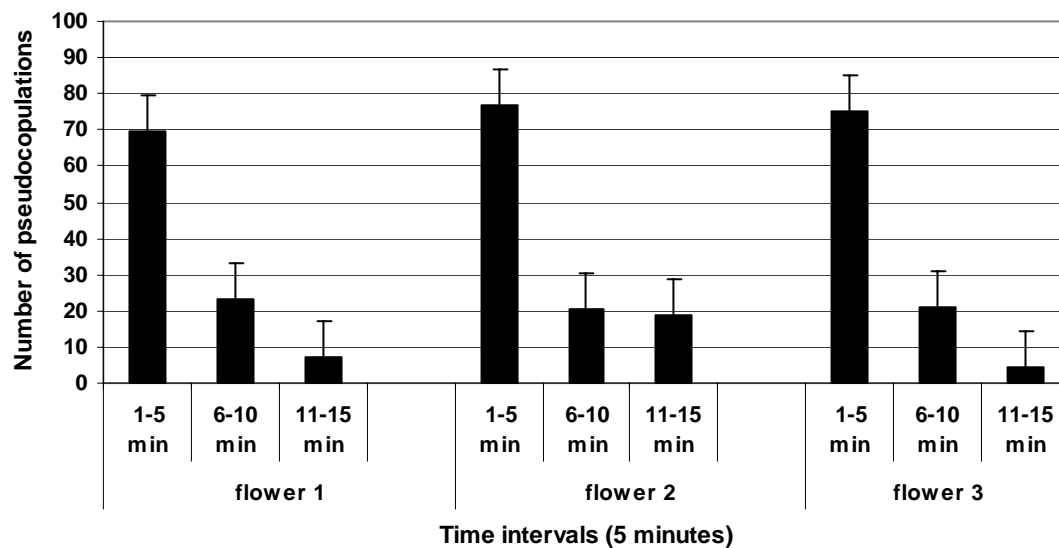


Fig. 3: Number of pseudocopulations of *Eucera nigrilabris* with 3 flowers of 3 different plants of *Ophrys tenthredinifera*. After 15 minutes nearly none of the flying males had interest on the presented flower. Presented a new flower nearly all males came instantly back for copulations. The same happened with a third new flower. Observations in Tunisia, March 1999, sum of 3 experiments, n=412 landings.

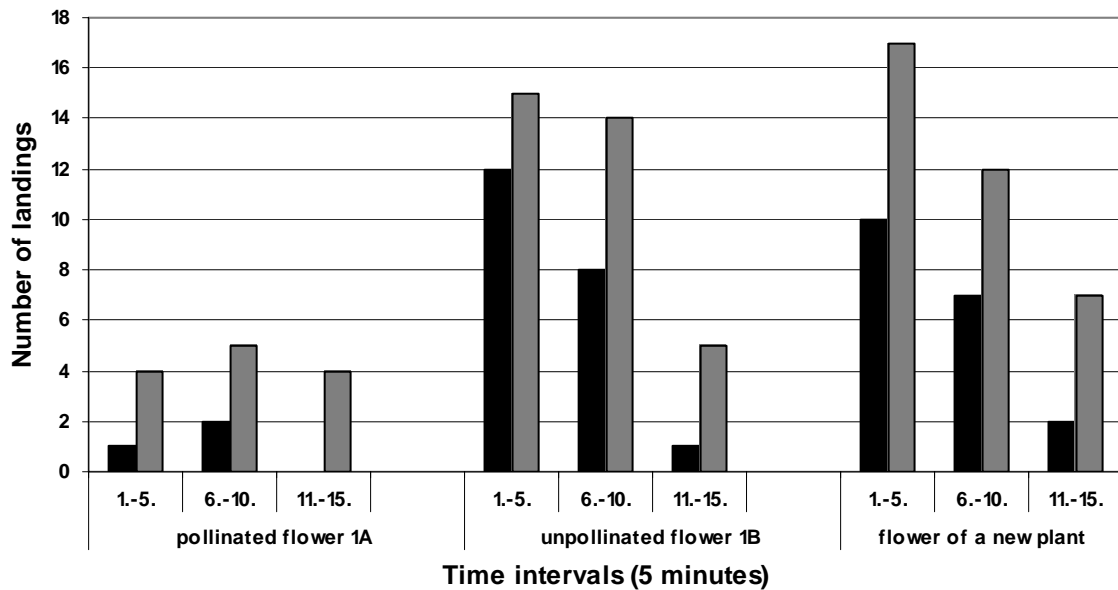


Fig. 5

Choice experiments with *Eucera nigrilabris* on *Ophrys tenthredinifera*: Males differentiate between pollinated and unpollinated flowers: First a pollinated flower (flower 1A) was presented, after this an unpollinated flower (flower 1B) of the same plant individual. The pollinated flower was clearly unattractive in comparison with an unpollinated flower of the same plant or a new flower of another plant individual. Experiments in Tunisia, March 1999, sum of 2 experiments, n=126.

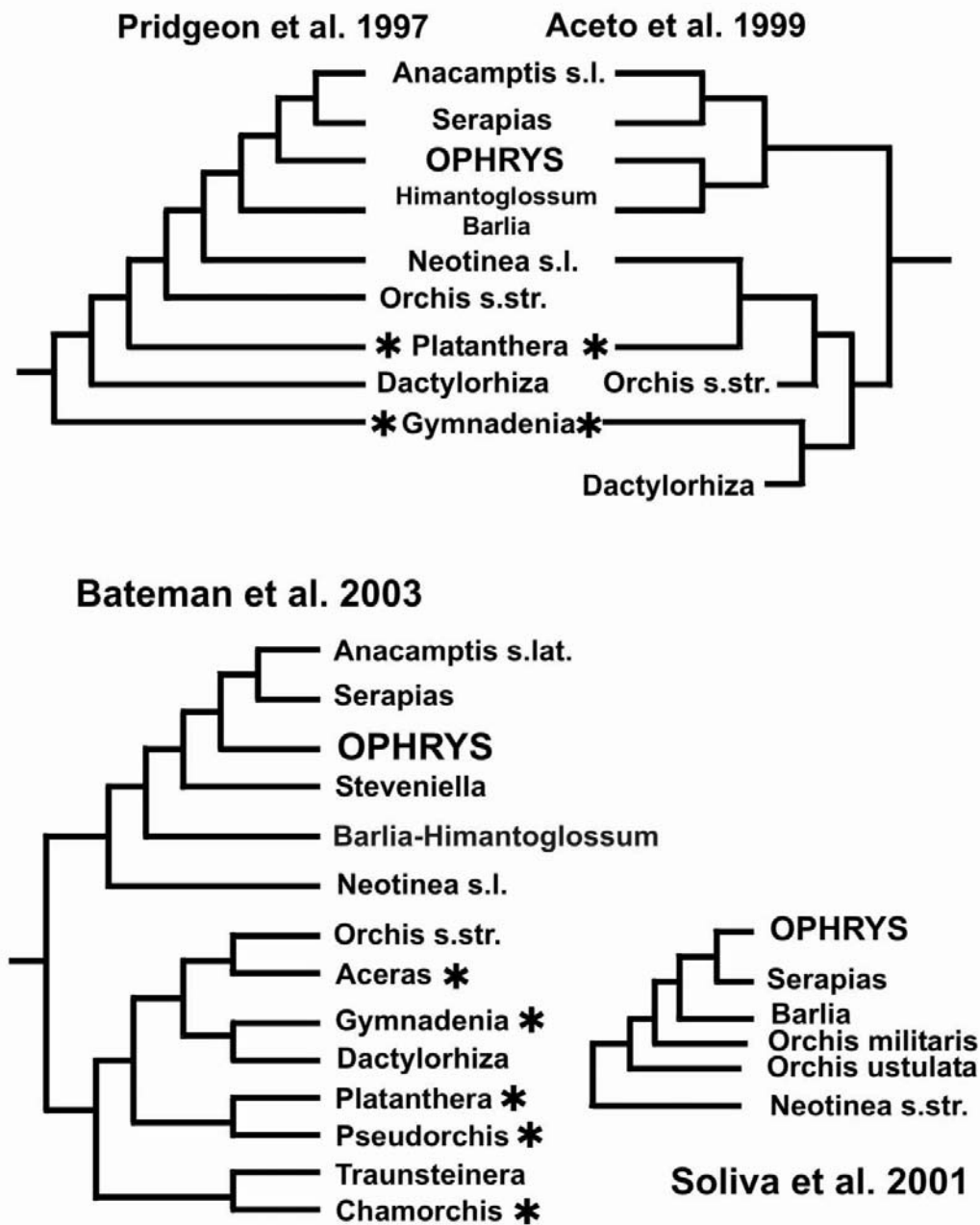


Fig. 6

Comparison of molecular dendrograms of the Orchidinae from different authors. The phylogenetic position of *Ophrys* is unsure. The genus is either a sister group of *Barlia-Himantoglossum* (ACETO et al. 1999) or of *Serapias* + *Anacamptis* s.l. (PRIDGEON et al. 1997, BATEMAN et al. 1997, SOLIVA et al. 2001, BATEMAN et al. 2003). All genera marked with an asterisk produce nectar for attracting pollinators. In all dendrogram variants we can suppose that the sexual deception mechanism of the genus *Ophrys* derived from a nectar deceiving precursor.

Figures (Colour table pag. 353)

1	2
3	4

Colour Fig. 1

Flower of *Ophrys vernixia* besides the pollinator female *Dasyscolia ciliata* (Hymenoptera, Scoliidae): Remark the striking optical similarity which regards to the main hair colour, the blue shining labellum mirror in comparison with the same shining of the female's wings or even the the lateral lobes of the labellum which imitate the hind legs of the female wasp (Foto: H.F.Paulus, 18.3.1976, Marbella, South Spain).

Colour Fig. 2

Example for head pollination in *Ophrys episcopalis* with its pollinator, the long-horned bee male *Eucera (Synhalonia) cressa* (formerly named *Tetralonia cressa*) (Apoidea, Anthophoridae, Eucerini) (Foto H.F.Paulus, Tripti 2.5.2002, East Crete, Greece).

Colour Fig. 3

Example for abdominal pollination in *Ophrys lindia* Paulus with its pollinator, the sand bee *Andrena (Truncandrena) truncatilabris* (Apoidea, Andrenidae) (Foto H.F.Paulus, 19.4.2001, southerly Kattavia, South Rhodes, Greece).

Colour Fig. 4

Eucera (Synhalonia) berlandi (Tetralonia berlandi) (Apoidea, Anthophoridae, Eucerini) is the pollinator of *Ophrys heldreichii* (Foto H.F.Paulus, 6.4.1982, Gournia, East Crete, Greece). We made many experiments with these male bees in Crete.

