"DYNAMIC SPECIATION PROCESSES IN THE MEDITERRANEAN ORCHID GENUS *OPHRYS* L. (ORCHIDACEAE)"

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Cover picture: Pseudo-copulation of a ***Colletes cunicularius*** male on a flower of ***Ophrys exaltata ssp. archipelagi*** (Marina di Lesina, Italy. H. Breitkopf, 2011).

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GENERAL INTRODUCTION

ORCHIDS

With more than 22.000 accepted species in 880 genera (Pridgeon et al. 1999), the family of the *Orchidaceae* is the largest family of angiosperm plants. Recently discovered fossils document their existence for at least 15 Ma. The last common ancestor of all orchids has been estimated to exist about 80 Ma ago (Ramirez et al. 2007, Gustafsson et al. 2010). Orchids are cosmopolitan, distributed on all continents and a great variety of habitats, ranging from deserts and swamps to arctic regions. Two large groups can be distinguished: Epiphytic and epilithic orchids attach themselves with aerial roots to trees or stones, mostly halfway between the ground and the upper canopy where they absorb water through the velamen of their roots. They are typically found in tropical forests of South America, Central Africa and South-East Asia. Terrestrial orchids constitute the other large group of orchids. Their supply with water and nutrients relies on underground roots or rhizomes. Many species form one or more specialized repository organs (root tubers); their shape is where the families' name is derived from (gr. ορχισ = testicle). They are generally distributed outside the tropics, mainly in temperate and sub-tropical regions of Eurasia, Australia, North America and South Africa.

BIOLOGY AND LIFE CYCLE OF TERRESTRIAL ORCHIDS

Eurasian distributed orchids are perennial herbs that produce underground rhizomes or tubers to persist the period of hibernal dormancy. The roots are mostly short, thick and sparsely ramified. Additional to sexual reproduction, many rhizomatous species are able to propagate via vegetative growth and build up large clonal patches (e.g. *Epipactis palustris*, *Cypripedium calceolus*) while only very few tuberous species generate additional root tubers (e.g. *Serapias lingua*, *Ophrys bombyliflora*).

Tuberous species have a characteristic life cycle: During the vegetative period the under ground repository tuber is used up to generate above ground organs from leaves to fruits, while contemporaneously a new tuber is developed to persist the dormancy period. In the Mediterranean basin the leaf rosette is usually developed in the winter period, but this can be delayed until the early

summer, depending on the species and the vertical distribution in Europe. *Spiranthes spiralis* largely deviates from this pattern: The leaves develop in autumn, and they wither until the late summer, when the inflorescence arises beneath the rosette. *Goodyera repens* is the only evergreen species. Most orchids flower from early spring to the middle of summer, fewer species from late summer to autumn (e.g. *Epipactis* spp.). The plants form only a single unbranched (monopodial) erect and racemose inflorescence with generally resupinate flowers. Rarely the labellum points upwards (e.g. *Nigritella* spp.). The flowers of the European Species follow the general pattern described above but display a great variability regarding shape, color and pollination syndromes. As in *Asclepiadaceae* too, the pollen forms a coherent mass that is dispersed as a single unit during pollination. These so-called pollinia are attached with a viscid disc to different parts of the pollinators' body (Darwin 1862).

Orchid flowers typically have a bilateral-symmetric perianth consistent of two circles with three tepals each. The upper, middle tepal of the inner circle is usually very differentially shaped. Through resupination in most species it points downwards and plays a prominent role in the attraction of pollinators and as landing platform. This highly specialized floral organ is an autapomorphy of the *Orchidaceae* and called 'labellum' or 'lip'. Primitive orchids have 3 stamens, *Apostasia* and the *Cypripedioideae* have two stamens. All other orchids have only one stamen with the other two being reduced to staminodia. The filaments are fused with the style. They form a typical organ, called column. The column also forms the rostellum, a tissue separating the anther(s) from the stigma und thus preventing self-fertilization. It can also function as a sort of bag containing the adhesive discs of the stalked pollinia.

Orchids have tricarpellate, inferior ovaries. Fertilized flowers produce fleshy capsules with thousands of dust-like seeds (except some members of *Vanilloideae* and *Cypripedioideae*) that can be transported with the wind over long distances, though mostly in a distance of a few meters. An air-filled coat surrounds the small embryo. The low weight of the seeds is due to the lack of a rigid testa and a nutritive tissue (endosperm) that feeds the developing plantlet until the beginning of the photosynthetic phase (Jersakova & Malinova 2007). All orchids are myco-heterotrophic, i.e. in the germination and seedling stage they are dependent on basidiomycetic fungi that deliver nutrients for root growth and leaf development. But some orchid species retain the fungal symbiont throughout their lives: e.g. *Neottia nidus-avis* and *Corallorhiza trifida*. They are saprophytic

orchids that lost the ability to do photosynthesis. Specificity and properties of orchid-mycorrhiza symbiotic relationships are hardly explored yet (Rasmussen 2002).

BREEDING SYSTEM

Most Eurasian species are allogamous with, compared to tropical orchids, relatively inconspicuous small flowers. But some taxa changed their breeding system and have become autogamous (e.g. *Ophrys apifera*, *Epipactis helleborine* agg. p.p., see: Squirell et al. 2002, Hollingsworth et al. 2006), cleistogamous (e.g. *Neotinea maculata*) or they flower occasionally underground (e.g. *Neottia nidus-avis*, *Epipogium aphyllum*). The spectrum of pollinators ranges from small flies over ants, all kinds of bees and wasps, to beetles and butterflies, amongst others. While generally a reward in form of nectar is offered, some species and genera evolved food- and sexually deceptive flowers that offer no reward at all to the pollinators. Food-deceptive species are numerous in *Dactylorhiza* and *Orchis s.l.*. These have floral traits typical for rewarding species, but they tend to flower earlier than sympatric rewarding species (Pellissier et al. 2010), they are accompanied by rewarding 'magnet plants' (Johnson 2003) or mimic rewarding species (Dafni 1981) to overcome the overall lower attractiveness to pollinators. Other forms of mimicry in orchids include mimicry of pollen (*Calypso bulbosa*, see: Boyden 1982), green leaf volatiles (*Epipactis* spp., Brodman et al. 2008) or alarm pheromones (*Epipactis veratrifolia*, see: Stökl et al. 2011). All species of the genus *Ophrys* are sexually deceptive, mimicking morphological characteristics and pheromones of female insects. This mechanism likely exhibits the most astonishing case of mimicry in orchids. The genus also depicts the only occurrence of sexual deceptive plants in Europe and the Mediterranean Basin.
Many orchids have developed highly specialized pollination systems. Hence fertilization can be rare. This is thought to be the reason for the relatively long receptivity of their flowers, the delivery of pollen as a unit, and the high number of produced seeds.

ECONOMICAL USES

Many species of tropical epiphytes, including their hybrids are cultivated as ornamental plants on a large scale (e.g. *Phalaenopsis*, *Epidendrum* and *Cattleya*).

Besides that, only few reports for medicinal applications or use as food have been reported: The root tubers of several *Orchis s.l*. spp. ('Salep') are used as tee herbs and some *Epidendrum* spp. are employed in traditional Chinese medicine (see also: Bulpitt 2005). The dried fruits of some *Vanilla* spp., mostly *V. planifolia*, are commonly used as flavouring. The worldwide yield of 'Vanilla' in 2006 was as high as 10.5 tons, with Madagascar being the major producer.

TAXONOMY

The *Orchidaceae* have been earlier attributed to three different families (Dahlgren 1985), but they later turned out to be polyphyletic. Based on recent molecular and morphological analyses the *Orchidaceae* have been reunited and are now placed in the order *Asparagales* (APGIII 2009) as sister to the remainder of the clade (incl. *Hypoxidaceae s.l.*, *Iridaceae*, *Asphodelaceae*, *Alliaceae*, *Amaryllidaceae*, *Agavaceae*, etc.). Within the *Orchidaceae* five subfamilies are recognized (Freudenstein et al. 2004): the basal *Apostasioideae* (3 stamens) and the more derived *Cyprepedioideae*, *Vanilloideae*, *Epidendroideae* and *Orchidoideae* (all monandrous). Most European distributed orchids come from the subfamilies *Epidendroideae* (e.g. *Epipactis*, *Listera*, *Neottia*) and *Orchidoideae* (e.g. *Orchis s.l*., *Dactylorhiza*, *Platanthera*).

OPHRYS

Within the *Orchidinae* the orchid genus *Ophrys* is morphologically distinct and genetically well defined (Bateman et al. 2003). *Ophrys* species are perennial herbs that form a basal rosette in the late autumn and a single few-flowered inflorescence in the time of late winter till early summer. Within the vegetative period the underground repository tuber is used up to generate above ground organs, while contemporaneously a new tuber is developed to persist the dormancy period. A large-scale demographic study on *O. sphegodes* (Hutchings 2010) revealed medium life spans of 2.25 years, with few individuals living as long as 20 years. 30% of plants had dormancy periods of (1—) 2 (—4) years.
Like closely related genera, *Ophrys* spp. are generally diploid with a basic chromosome number of 2n=36. For a few taxa, mainly of subg. *Pseudophrys* diverging chromosome numbers, as well as different ploidy levels have been reported (Bernardos et al. 2003, D'Emerico et al. 2005, Vereecken et al. 2010).

POLLINATION BY SEXUAL DECEPTION

Floral features of animal pollinated flowers are the consequence of a co-evolutionary process in which the plants generally adapt to the pollinators preferences for food and evolve visual, tactile and structural features that both attract the pollinator(s) and exclude unwanted visitors. A pollination syndrome is a suite of floral features that evolved under natural selection imposed by one or more pollinators (Fenster et al. 2004). Due to the complexity of extant plant flowers, relationships between monophyletic entities are often correlated with the pollination syndromes. In contrast to the majority of plant species, no reward in the form of nectar or pollen is offered in food-deceptive and sexually deceptive species (Jersakova et al. 2006).

The genus *Ophrys* is characterized by pollination through sexual deception, which was until recently only known for *Orchidaceae* spp. (Ciotek et al. 2006); but Ellis & Johnson (2010) reported a sexually deceptive daisy from South Africa. *Ophrys* orchids mimic morphological features and the female sexual pheromones of certain bee species (rarely wasps or beetles) to deceive inexperienced male bees into landing and copulating on the flower labellum. The pollinia get attached to the head or abdomen of the male bee, which will likely repeat the so-called pseudo-copulatory behaviour on another individual, and in this way effectuate cross-pollination (Pouyanne 1917). Each *Ophrys* species is pollinated by only one or very few bee species (Lorella et al. 2002, Gaskett 2010). This pollination system is thought to be highly effective and species specific (Kullenberg 1961), mainly due to the floral scent which is emitted from the labellum and to a minor degree also from other parts of the flower: differential pollinator attraction is based on a composition of various semio-chemicals, mostly alkanes and alkenes or, within closely related species, often alone on the relative proportions of these (Schiestl et al. 1999, Mant et al. 2005) or the position of double bonds in pollinator attracting alkenes (Schlüter et al. 2011).

The model female bee is somewhat simulated by a combination of olfactory, visual and tactile cues. After the floral scent has guided the pollinator to the flower (long-distance attraction; see: Ayasse et al. 2003), the function of the morphological structure of the labellum comes into the game. The role of the different components of the complex morphological floral equipment has been insufficiently investigated for long time. Recent studies highlighted the

importance of visual cues like petal color and speculum structure (Streinzer et al 2009, 2010; Spaethe et al. 2010) for pollinator attraction at short distances. The highly reflective speculum has been interpreted as a mimic of hymenopteran wings that also reflect the light. Trichomes can be found on nearly all *Ophrys* spp. labella (Bradshaw et al. 2010). They are reputedly mimicking the female insects' hairs and their direction is leading the pollinator into the right position, i.e. pollinia take-up and delivery. Distally aligned hairs force the bee to take up a head-up position on the lip (subg. *Euophrys*), while proximally aligned hairs affect the bee to assume a head-down position (subg. *Pseudophrys*). Also the three-dimensional topology of the lip guides the insect to find the right position for pollination.

FLORAL ISOLATION

Floral isolation and pollinator shifts are well documented for a variety of plants, e.g. *Mimulus* (Bradshaw & Schemske 2003), *Aquilegia* (Whitall & Hodges 2007), *Anacamptis* (Dafni & Ivry 1979) and *Ophrys* (Kullenberg 1961, Paulus & Gack 1990, Schlüter et al. 2007). Because *Ophrys* species are interfertile, reproductive isolation in this genus mainly relies on floral isolation caused by high pollinator specificity. Sexually deceptive plant systems are characterized by strong pre-zygotic and weak post-mating isolation barriers. Pollinator shifts are thought to happen fast (Cozzolino & Widmer 2005). Therefore the potential for speciation and the built-up of reproductively isolated populations and new species is high compared to generalized, rewarding and food-deceptive taxa (Scopece et al. 2007).

Though, floral isolation was shown to be acting almost perfect in a group of closely related *Ophrys* spp. (Xu et al. 2011), hybrids and hybrid zones can be met (Souche 2008, Cortis et al 2008, Stoekl et al 2009). Crossing barriers are virtually absent (Ehrendorfer 1980, Scopece et al 2007). So far, post-zygotic isolation could only be demonstrated for backcrosses of triploid hybrids with the di- and tetraploid parental species (Vereecken 2010). Thou, recent experiments demonstrated the floral isolation of the investigated taxa to be leaky (unpubl. observation 2011). This raises the question whether hybrids backcross with the parents, and if so, how much gene flow is tolerated without breaking down species boundaries.

DIVERSITY & TAXONOMY

Hotspots of *Ophrys* diversity are found in France, Italy and Greece, but the distribution area of the genus ranges from the Canary Islands to Iran and from Scandinavia to North Africa. According to the many taxonomic treatments available, 17 spp. (Sundermann 1980) to more than 250 spp. (Delforge 2006) are accepted. The genus is traditionally divided in two subgenera: 1. subg. *Pseudophrys*, where the pollinia are placed on the abdomen of the pollinator, and 2. subg. *Ophrys* (syn. *Euophrys*), where the pollinia are placed on the pollinators head. Still, many new species are described every year on the basis of minor morphological differences, lacking any significance due to missing pollinator records. This contrasts with the widely accepted fact that floral morphological characters between and even within populations of a given species can vary notably. Therefore the morphological species concept cannot always be applied with confidence to closely related taxa (Vereecken et al 2010). Interestingly, all of the more widely distributed *Ophrys* taxa are believed / have been demonstrated to be relatively old species at the base of the tree and/or at the core of the more diversified Mediterranean groups, e.g. *O. bombyliflora*, *O. apifera*, *O. speculum*, *O. tenthredinifera*, *O. insectifera*, *O. sphegodes* and *O. holoserica*. Most of these locally adapted to novel pollinators and became reproductively isolated from their ancestors, or at least replaced them locally due to the predominance of the novel pollinator or the absence of the ancestors' pollinator. The mechanism involved in this adaptive speciation process is likely a selective pressure imposed by a novel pollinator, in which the flowers and their scent bouquet evolve towards the preferences of the insect. In contrast to this creeping process involving only minor changes in a continuous way, mutations in genes responsible for the production of scent components, as well as the formation of hybrids exhibit great potential for the recruitment of novel pollinator species.

The most species rich complexes of the genus recruited different groups of bees: The *O. holoserica* group is mainly *Eucera* pollinated, while the *O. sphegodes* group is predominantly *Andrena*/*Colletes* pollinated. The *O. fusca* group recruited a similar set of pollinators as the *O. sphegodes* group, but the species of the two groups are mechanically isolated through the differential pollen placement on the insect body. Several minor groups have been described based on similarities of morphological features and the respective pollinators, e.g. *O. bertolonii* group

(*Chalicodoma* pollinated), *O. lunulata* group (*Osmia* pollinated) and *O. argolica* group (*Anthophora* pollinated) (Delforge 2006). It is unknown whether these groups are natural units, i.e. monophyletic, because all of their defining characteristics are not exclusive to them.

SPECIES CONCEPTS

In biology the species is traditionally seen as the basic evolutionary unit (Mayr 1982). Though, the question of how to define a species constitutes a long-standing debate among biologists. The debate itself has become known as the "species problem" (Mayr 1957, Hey 2001). The problems associated with finding a universally valid definition are not only based on empirical grounds but also on theoretical appraisements and the perception of the subjective individual (Gilmour 2008, Hey 2001). The aim is to define a fuzzy-boarded entity, part of a continuum from individuals, populations, over varieties and subspecies, to genera.

Providing a definition that is adequately applicable to all kinds of organismal groups seems impracticable if not impossible, especially when looking at groups as different as bacteria, fungi, animals and plants. "No one definition has satisfied all naturalists; yet every naturalist knows vaguely what he means when he speaks of a species", Darwin quoted. He viewed speciation as a gradual process and used the term species in the sense of a provisional name tag for interbreeding organisms: "I look at the term species as one arbitrarily given for the sake of convenience to a set of individuals closely resembling each other".
Traditionally, species were identified by comparative anatomy and morphology ('classical species', 'phenetic concept', 'morpho-species'; Linne: see Larson 1968; Cronquist 1978), and the grade of variation was used as a measure for phylogenetic relatedness [uncertainties about species membership are displayed in the use of the terms 'subspecies' and 'variation'].
Poulton (1904) was the first to outline the importance of reproductive isolation (syngamy vs. asyngamy) in an inclusive species delimitation, but Theodosius Dobzhansky and Ernst Mayr are jointly quoted for their modern evolutionary syntheses and the 'biological species concept' (BSC). They similarly defined a species as "… that stage of evolutionary progress at which the once actually or potentially interbreeding array of forms becomes segregated into two or more

separate arrays, which are physiologically incapable of interbreeding" (Dobzhansky 1937) and as "groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups" (Mayr 1942), respectively. Based on the inclusive BSC, more stringent concepts came up. The 'ecological species concept' draws the line between species, even with ongoing gene flow, if they are ecologically distinct (Van Valen 1976). Different to that, the 'phylogenetic concept' (or 'diagnostic concept') by Cracraft (1989) defined a species as "an irreducible (basal) cluster of organisms, diagnosable distinct from other such clusters, and within which there is a parental pattern of ancestry and descent". But this strict definition has its shortcomings: Good species and its' populations may or may not be resolved in a phylogenetic tree. This depends on the evolutionary rates of the employed genetic markers, as well as the divergence times of the analyzed lineages, i.e. populations of one species may be resolved, but an array of reproductively isolated species may be unresolved, using the same genetic tools. This means that levels of resolution in a phylogenetic trees does not necessarily allow for any inferences regarding the species status. Less inclusive (diagnostic) concepts for plants generally allow for gene flow between species, i.e. they don't have to be reproductively isolated, if "the lineage evolves separately from others with its own unitary evolutionary role and tendencies" (Simpson 1951).

The BSC is historically the most quoted concept in biology, and most of the other proposed definitions are conceptionally related to the BSC. A general criticism in the BSC is the practical inapplicability of the concept: artificial crosses and checking the offspring's' fertility may be inconclusive due to the exclusion of many environmental factors, or infeasible in terms of money and time. Furthermore, the BSC is not applicable to many lineages of land plants: interspecific hybridization between clearly delimited species and the existence of autogamous lineages are common phenomena in angiosperms (Mishler 1985, Cronquist 1988).

A different approach is to identify the evolutionary significant unit (ESU). The individual and his genes are the smallest possible unit, but the evolution of new species necessarily involves other individuals, generations of recombination and inheritance, and of course, some kind of isolation mechanism. Therefore, "populations are the real units of evolution" (Ehrlich & Raven 1969). This view is shared by conservational biologists who seek to protect ESU's (Fraser &

Bernatchez 2001). These can be species, by whatever definition, and lower taxonomic units.
The definition of a valid species concept for a given group of organisms ideally has to take into account morphological variability, phenology, distribution, interfertility between populations and closely related species, fertility of the offspring and the composition and the strength of all reproductive isolation barriers. As it is an almost impossible exercise to answer all these questions satisfactorily, researchers tend do rely only on some components of the above-mentioned concepts, such as reproductive isolation, phylogenetic resolution, ecological distinctness, etc. to define "their" species, which is a highly subjective choice.

De Queiroz (1998) argued, that all species concept provide criteria only applicable to a certain stage of the speciation process, and that all known species concepts agree on the fact that "species are independent lineages". However, this "general lineage concept" factors out all problems associated with the splitting process itself and is therefore not helpful for the validation of lineages with gene flow.

The lack of a definite species concept poses practical problems to taxonomists. The spectrum of possible taxonomic treatments is outlined by two opposed approaches: lumping and splitting. Lumpers use a more inclusive concept (e.g. BSC) that accepts the existence of polytypic species, i.e. a number of subspecies within one big species. Species should keep up their (genetic) integrity, even in sympatry. Lumpers legitimate their use of a more conservative and inclusive concept with the fact that many factors influencing a species and its integrity are unknown. In contrast to that, splitters generally use one of the more diagnostic and strict concepts available that allow for gene flow between separate species, if these taxa have their "own evolutionary role and tendencies" (Simpson 1951).

As for the genus *Ophrys*, there is still a spirited debate if (1) morphologically slightly dissimilar taxa that share one pollinator species should be treated as different diagnostic species when growing in parapatry/allopatry (e.g. *O. exaltata s.l.*), if (2) morphological nearly identical taxa with different pollinators (cryptic species) should be treated as a single polytypic species (e.g. *O. fusca s.l.*, see: Schlueter et al. 2011), and if (3) genetically barely distinguishable taxa with

similar morphology should be treated as polytypic species (Pedersen & Faurholt 2007, but see: Bradshaw et al 2011, Vereecken et al. 2011).

AN ETHOLOGICAL SPECIES CONCEPT FOR OPHRYS

Based of the 'evolutionary species concept' (Simpson 1951) and the 'ecological species concept' (Van Valen 1076), a species can be defined as an evolutionary unit occupying an ecological niche and an evolutionary role different from any other closely related lineages. The only in deep investigated strong isolation barrier in sympatric *Ophrys* is floral isolation imposed by different pollinators (Xu et al. 2011). Therefore a modified ecological/evolutionary species concept for *Ophrys* necessarily involving the mechanism of isolation by the pollinating insects could be named 'ethological species concept'.

There is a steadily growing consensus among *Ophrys* researchers that an 'ethological species concept' seems most appropriate for the discrimination of species. This concept means, that a species is defined by having mostly only one single pollinator; it includes all morphologically similar populations (varieties); morphologically nearly identical populations with different pollinators are belonging to different species. But morphologically largely dissimilar populations (mechanical isolation through differential pollinia placement or great size differences) are different species, even if they share a pollinator. In short, 'species' can have gene flow, if they occupy an adaptive zone different from other lineages (Van Valen 1976) and if they have an own evolutionary role (Simpson 1951).

Problems for defining a species in *Ophrys* result from the difficult cases, where taxa/populations are minimally different from each other in terms of morphology and/or where reproductive isolation based on different pollinators can be leaky due to erroneous/illegitimate pollination or overlap in the spectra of possible pollinators. Moreover the pollinator record for almost all *Ophrys* species is fragmentary or missing. This is not surprising at all, as compiling a 'really complete record' would include field observations over the whole flowering period, repeated in subsequent years; doing choice experiments with sympatric species, preferentially also translocation studies, etc.

As shown with choice experiments in sympatric Italian *Ophrys* populations (unpubl. observations 2011), closely related taxa keep up their integrity even in the presence of pollinator overlap and little gene flow (Xu et al. 2011). Even though the future of these overlapping lineages is uncertain, they clearly represent evolutionary significant units with the potential to become more strongly isolated with time. In the authors view, the presence of interfertility and the genetic similarity within close relatives in *Ophrys* doesn't justify lumping them together as polytypic species. This practice is very imprecise, because nobody knows whether the populations/subspecies/varieties of a polytypic species constitute a monophylum, or if so, whether they will stay monophyletic over time. It seems more accurate to judge the ESU's as the most important category: these can be varieties, subspecies or even 'good species'. Examples:

1. *O. archipelagi* and *O. tyrrhena* are morphologically similar, they share one pollinator species, but they are distributed on different, opposed sides of the Italian peninsula. The two taxa could be treated as diverging populations of only one lineage, i.e. a **variety**.
2. *O. sphegodes* and *O. argentaria* are morphologically and genetically very similar, but they have, as far as it is known, different pollinators and a distribution like the above given example. These taxa could be treated as different **species**, as long as no pollinator overlap is found.
3. *O. sphegodes* from Gargano (Puglia, Italy) and from the province of Naples (Campania, Italy) have been treated as belonging to the same species, even if minor but constant morphological differences characterize these differentially distributed demes. It has been found that the two populations have different pollinators. But at least the plants from Naples attract to a minor degree the pollinator of the plants from Gargano. This most likely represents a local adaptation of *O. sphegodes* to a novel pollinator, with incomplete reproductive isolation from the ancestral species. Both lineages have their own ecological and evolutionary attributes, but they would largely hybridize in sympatry. The term **subspecies** explains best the parentage of the ancestor and the distinctness due to a pollinator switch and slight morphological divergence.
4. In *Ophrys*, *O. speculum* represents the rare case of a '**good species**'. It is pollinated by only a single wasp species that is not shared with any other *Ophrys* lineage. Occasional hybrids with other Ophrys species can be found, though.

Alone the presence of a major pollinator, isolating a lineage to a high degree from other sympatric lineages, gives the taxon in question a unitary evolutionary potential that may lead to total reproductive isolation in the future. One should be aware that we are looking at a snap-shot of the speciation of incipiently diverging lineages, when trying to delimit *Ophrys* species.

As a taxonomist it seems adequate to treat incipient lineages as polytypic species, because phylogenetic relationships are to some degree reticulated and divergence is incomplete. This means the observed pattern might be well different in the future.

From an evolutionary biologists point of view a polytypic species concept overlooks all the processes and different stages from early divergence to total isolation. As the taxonomist too, he needs to use categories.

GENE FLOW & HYBRIDIZATION

Even though the 'ethological concept' is perhaps the best approximation to the task of defining an appropriate species concept for *Ophrys*, it is not waterproof. The increasing findings of additional, 'minor pollinators' for species, that previously have been thought being pollinated by a single pollinator, further complicates answering the tasks regarding a valid species concept. For example, for 5 out of 6 investigated species from the Gargano area in Italy at least one novel pollinator has been observed in choice experiments in the field (unpubl. observation, 2011). This finding suggests interspecific cross-pollination of species that were thought to be reproductively isolated from each other. As the *Ophrys* spp. in question can be distinguished in the field using morphology, it's likely that some gene flow exists without breaking down species boundaries. But this might be responsible for some allele sharing that can seriously hinder phylogenetic reconstruction, especially when parts of the genome are investigated, that are not under selection. Lineage sorting of genes under selection will happen relatively fast compared to genomic regions that are not under selection (e.g. introns). Other studies on *Ophrys* and Australian deceptive orchids found evidence for possible gene flow between closely related species (Mant et al 2002, Soliva & Widmer 2003).

REVIEW OF PREVIOUS PHYLOGENETIC STUDIES

In recent years, progress has been made in reconstructing familiar relationships within the *Orchidinae*, a Eurasian distributed subtribe of the Orchideae which comprises only terrestrial orchids (Aceto et al. 1999, Bateman et al. 2003). *Ophrys* was shown to be monophyletic and, as well as the sister genus *Serapias*, it is characterized by a relatively long branch, relative to all other taxa of the subtribe. Previously, two studies focused on the phylogeny of *Ophrys* (18 ingroup taxa: Soliva et al. 2001, Devey et al. 2008). Traditionally employed gene markers for phylogenetic reconstruction were used in these works: Internal transcribed spacer 1 and 2 (*ITS*), as well as the chloroplast markers *trn*L-*trn*F and *trn*H-*psb*A + *trn*D-*trn*T, respectively. Ten, well supported species groups were revealed by Devey et al. (2008), though the relationships between them could not be resolved in most cases or support was low. Notably the placement of the *O. insectifera* group to the remainder of the genus, the relationships between the *O. speculum*, *O. tenthredinifera* and *O. fusca* groups and those between the species rich *O. sphegodes*, *O. holoserica*, *O. scolopax* and *O. umbilicata* groups remained unresolved or insufficiently supported. No resolution was obtained towards the terminals of the tree. Devey et al. (2008) found heterozygous individuals in the ITS; the cloned alleles were mostly non-monophyletic, often grouping with different species groups. Alleles of the tetraploid *O. dyris* even grouped in both of the two subgenera. This finding was interpreted as the consequence of hybridization between more distant lineages. Even though not directly comparable, the results of AFLP's were similar to the *ITS* gene tree (Devey et al. 2008).

DATING THE AGE OF ORCHID LINEAGES

The age of the *Orchidaceae* and it's various lineages is of major interest for systematicists. Previously proposed ages for the family varied between ~26 Ma (Wikstroem et al. 2001) and ~110 Ma (Janssen et al. 2004). In recent years, the findings of three orchid fossils have enabled calibrations of the orchid phylogeny (Ramirez et al. 2007, Gustafsson 2010). The most recent common ancestor of the *Orchidaceae* has been estimated with a relaxed molecular clock (BEAST, Drummond et al. 2006, Drummond & Rambaut 2007) approach to exist around 76 - 84 Ma ago. The latest work using three calibration points and two plastid markers estimated the age of the *Orchidinae* to be ~15 Ma, based on single representatives of the two genera *Orchis* and *Platanthera*. The high number of

species in the genus *Ophrys* in conjunction with the observed low levels of genetic differentiation is thought to be the consequence of either a rapid radiation or past gene-flow (shared ancestral polymorphism)/ongoing introgressive hybridization, or both. Elucidating this task is essential for disclosing the forces driving the immense diversification of the genus *Ophrys*.

QUOTATION

"One should never quarrel about words, and never get involved in questions of terminology. One should always keep away from discussing concepts."

—Karl Popper, Objective Knowledge: An Evolutionary Approach

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"MULTI-LOCUS NUCLEAR GENE PHYLOGENY OF THE SEXUALLY DECEPTIVE ORCHID GENUS *OPHRYS* L. (ORCHIDACEAE)"

INTRODUCTION

The majority of phylogenetic questions in plant systematics is still addressed by use of relatively few molecular tools, namely sequence analysis of only one or very few loci from the chloroplast (cpDNA) or nuclear ribosomal genomes (rDNA)(Small 2004). The applicability of this approach can reach its limits when it comes to the analysis of recently diverging lineages: (1) Chloroplast loci are linked and do not recombine due to the genomes uni-parental inheritance. This means that all loci contain the same phylogenetic signal for a tree topology that must not necessarily reflect the real species tree. Furthermore, only a single haplotype is fixed within an individual organism and information about paternality and demographic history is lost. (2) Insufficient sequence variation can be limiting too, as both the chloroplast and the ribosomal genomes are small compared to the nuclear genome which, above that, has synonymous substitution rates five-fold higher than cpDNA (Wolfe 1987, 1989, Ossowski 2010). (3) Due to the likelihood of the occurrence of retained ancestral polymorphism, incomplete lineage sorting, and introgression among incipiently diverging lineages, gene genealogies of fast radiating groups are predicted to yield varying, incongruent topologies (e.g. Hey 1994, Ting 2000). Therefore the use of multiple unlinked loci will increase the chance to obtain a tree that reflects the underlying demographic history of the organismal group in question (Edwards & Beerli 2000). The nuclear genome presents a near infinite source of phylogenetic information. Its bi-parental mode of inheritance, an overall faster evolutionary rate and the higher likelihood that genes are unlinked, are advantageous properties. But the effort of time and money needed for the development of nuclear loci that are easily amplifiable over a given set of species, and the occurrence of heterozygosity and paralogy have severely hindered their regular use in molecular systematics of non-model organisms so far (discussed in: Sang 2002, Small 2004, Alvarez 2008). As consequence of these limitations, multiple gene phylogenies of plants are still a rare exception and their

importance for inferring the history of closely related plant species and populations has rarely been tested yet (but see: Marais 2011).

Orchids (*Orchidaceae*, Order: Asparagales) have fascinated biologists ever since (e.g. Darwin 1862). Their extraordinary floral diversity and species richness have inspired many studies about their familiar relationships, ecology, and pollination biology and are typically considered as prime examples of species radiation. The *Orchidaceae* are likely the most species-rich family in the plant kingdom, only challenged by the *Asteraceae*'s diversity. In the European Flora, particularly the genus *Ophrys* attracted the interest of 'orchidophile' amateurs, taxonomists and evolutionary biologists, likewise. The conspicuous, insect-like flowers mimic morphological characteristics and the female sex-pheromone of certain hymenopterans, mostly solitary bees. The emitted floral scent is a bouquet of various semio-chemicals, mostly alkanes and alkenes (Schiestl 1999). If a conspecific male is successfully deceived it will try to mate with the flowers labellum, a behaviour termed pseudo-copulation (Pouyanne 1917). In the iteration of this process pollinia can be removed and transported to the stigma of another plants flower. This remarkable plant-pollinator relationship is known as 'sexual deception'. With the exception of one daisy species from Africa (Ellis & Johnson 2010) this mechanism of pollinator attraction is only known from few orchid genera, mostly Australian ones (Ciotek 2006).

The specific attraction of only one or very few bee species (Lorella 2002, Gaskett 2010) is thought to guaranty proper intra-specific pollen transfer (floral isolation), and in this way effectuate reproductive isolation between sympatric taxa (Kullenberg 1961), that are lacking any inter-specific cytological crossing barriers (Ehrendorfer 1980, Scopece 2007). The formation of hybrids is also not prevented by different ploidy levels as *Ophrys* species are generally diploid (2n=36); few rare exceptions have been reported (Bernardos 2003, D'Emerico 2005, Vereecken 2010). Floral isolation has been shown to act near perfect as isolation barrier between some closely related sympatric *Ophrys* (Xu 2011). Beneath differing flowering time and spatial isolation, also mechanical isolation through size differences of the flower and differential pollinia placement on the insects' body can contribute to reproductive isolation in sympatry.
This high specific pollinator imposed selection and the demonstrated potential easy to shift to a novel pollinator (PNAS paper) by small genetic changes have

been supposed as a driving force generating the species radiation of this orchid group and the difficulties in its taxonomic treatment. Indeed, the taxonomy of *Ophrys* is reflected by largely varying numbers of species that are accepted by different authors: 16 spp. (Sundermann, 1980), 19 spp. (Faurholdt & Pedersen, 2007), 49 spp. (Baumann & Künkele, 1982), 150 spp. (Devillers & Devillers-Terschuren, 1994), or as much as 252 spp. (Delforge, 2006).

Previous molecular phylogenetic studies employed ribosomal nuclear internal transcribed spacers (ITS1+2) and chloroplast markers (matK??) to investigate interspecific relationships in *Ophrys* (Bateman 1997, 2003, Soliva 2001, Devey 2008). While monophyly of the genus is highly supported in all analyses, the internal clade resolution was basically lacking. Taken together, major species groups have been well defined, but the relationships between them are yet largely unresolved. Towards the terminals of the trees, no resolution within closely related taxa was achieved.

Lack of genetic differentiation can principally be the result of recent radiation or introgressive hybridization. These two different processes go often hand in hand, as reproductive barriers generally become stronger with increasing genetic differentiation. The contribution of gene flow to the evolution of the terminal clades, and connected to that, the nature of species boundaries in *Ophrys* have been sprightly debated (Vereecken 2011, Bateman 2011). The strong floral isolation found in sympatric populations can be taken as an argument for the hypothesis that most terminal lineages are reproductively isolated from each other and therefore represent ecological species. Conversely, the documented occurrence of hybrids and hybrid zones (e.g. Cortis et al. 2008), as well as the observed low genetic differentiation, could be interpreted as result of the fact that terminal lineages are not reproductively isolated enough to diverge as separate clades in phylogenetic analyses. Under this light, the genus *Ophrys* encompasses both rapid diversification and the challenge of inferring species borders and relationships thus representing a major challenge for molecular systematics. For such case study, a multi-locus nuclear gene phylogeny might allow to trace down character evolution and get deeper insights into an intriguing plant groups' evolution that is likely to be shaped by the repeated adaption to different novel pollinators.

Here we present a combined analysis of intron sequences from seven nuclear genes and nuclear ribosomal internal transcribed spacers (ITS); five of the gene markers were newly developed for this study. We apply these novel tools to make inferences on inter-specific relationships, biogeography and the evolution of flower-pollinator interactions in the orchid genus *Ophrys*. A resolved molecular phylogeny would allow us to trace down character evolution and provides us with deeper insights into the evolution of this intriguing group of plants that is likely to be correlated to, and shaped by the spatial and temporal occurrence of its species-specific pollinators.

The authors aim to address the following topics: (1) phylogenetic relationships between the major species groups, (2) biogeographic history, (3) gene flow vs. isolation, and (4) pollination system evolution.

MATERIALS & METHODS

For the present study, following an initial exploration of over 100 published and newly designed primer combinations a set of seven nuclear gene markers was assembled and applied to sequence analysis of 38 species of *Ophrys*, covering all ten species groups previously recognized by *ITS* analysis (Devey 2008). Notably, all cpDNA, and most nrDNA markers tested showed no sequence variation among closely related *Ophrys* species. Sequences were generated for the five newly developed gene markers (*ACS*, *BGP*, *CAD*, *FAD6*, *MYB*), for *LFY*/*FLORICAULA* and for *ITS*. *LFY* and *ITS* have been shown to be phylogenetically informative in *Ophrys* (e.g. Soliva 2001, Schlueter 2007), and are here combined with variably fast evolving markers to obtain support at the backbone of the tree, as well as within terminal lineages. We used the same primers for PCR and sequencing, besides for leafy where sequencing was done with nested primers as described in Schlüter et al (2007). A list of all employed primers is given in Tab.3.

PRIMER DESIGN, HOMOLOGY ASSESSMENT & MARKER EVALUATION

An EST-library from floral cDNA's of *Ophrys sphegodes* has been built up by colleagues of the Dept. of Systematic Botany (University of Zürich, Switzerland). The EST's were assembled automatically with alignment software; the length of

the gathered sequences varied from ~100bp to 1800bp. BLAST searches in public databases (e.g. NCBI, TAIR) allowed in many cases an ascertainment of the genes identity or an approximation in the sense of high sequence similarity to known genes. Sequences of genes that have been useful, i.e. phylogenetically informative in other studies, were chosen preferentially as candidates for primer design. The EST's contained only coding, exonic sequence. To assess the intron-exon structure and identify highly conserved regions for primer design, the EST's have been aligned with BioEdit Vers.7.0.9.0 (Hall, 2001) to that single, or in most cases multiple genomic sequences that were available in online databases, and showed the highest similarity (e-Value) with the EST in question. Where possible, genomic sequences from more closely related taxa (orchids, rice, asparagus) were included in the alignment. Primers have been designed in the exonic regions flanking one or more introns, depending on their size predicted by the alignment. As intron sizes vary notably within taxa from different families and genera, the amplicon size often deviated largely from the prediction. To facilitate later applications an amplicon size of 300-900bp length was aspired. Primer design was done manually or using the online software 'Primer3' Vers.0.4.0 (Rozen & Skaletsky, 2000). Primers were checked for their expected annealing temperatures, hairpins and loops with 'OligoCalc' (Kibbe, 2007). Amplification was tested for different annealing temperatures. Primers that amplified multiple products were discarded. Single band PCR products were sequenced and compared to published sequences in gene bank. After confirmation that the primers amplified the gene they were designed for, sequence variability and cross amplification was checked for closely, and also more distantly related taxa. To assess exon/intron structure and proof homology of the PCR products, new sequences were aligned with those used for the primer design. A list of the markers selected for this study is given in Tab.1.

Tab.1 – Markers used in this study. Supposed gene function, length and primer annealing sites in the exon

Gene	Sequence length [bp]	Length aligned [bp]	Name / function of sequenced gene	Primer position in exon
ACS	663 - 708	718	Long-chain acyl-CoA-synthase-like	E6/E8
BGP	806 - 1008	1020	Beta-galactosidase-like	E14/E17
CAD	259 - 303	304	Cynnamyl alcohol dehydrogenase	E1/E3
FAD	427 - 505	505	Fatty acid desaturase	E3/E5
ITS	628 - 634	640	Ribosomal internal transcribed spacer	E1/E2
LFY	1924 - 2519	2656	*LEAFY*/*FLORICAULA*	E1/E2
MYB	143 - 147	147	Myb transcription factor-like	???

SAMPLING & DNA-EXTRACTION

Fresh leaf tissue or flowers have been collected in the field, and either directly stored in silica gel, or fresh at -20°C. A list with all species accessions, sampling locations and collectors is given in Tab.2, while pictures of the sampled taxa are displayed in Fig.1. DNA was extracted with a commercially available kit (GenElute Plant Genomic DNA miniprep kit, Sigma) or, for higher yields of DNA with a modified CTAB extraction method (Doyle & Doyle, 1990).

Tab.2 – **Sampled *Ophrys* taxa**, incl. sampling site, sampling date and the respective collector(s). 37 ingroup taxa, 1 outgroup taxon.

Species	Sampling site	Date of collection	Collector
O. apifera	Cilento, Campania, Italy	Jun-09	H. Breitkopf
O. arachnitiformis	France	Apr-03	N. Vereecken
O. archipelagi	Capoiale, Gargano, Puglia, Italy	Mar-09	H. Breitkopf
O. attica	S-Elassona, Cyprus, Greece	May-02	P. Schlüter
O. aymoninii	La Pezade, Larzac, France	May-10	R. Romolini
O. bertolonii	Mte. Veneretta, Sicily, Italy	Apr-09	H. Breitkopf
O. bombyliflora	Mte. St. Angelo, Napoli, Italy	Apr-09	H. Breitkopf
O. crabronifera	Caserta, Campania, Izaly	Apr-09	H. Breitkopf
O. cretica	NW Spili, Crete, Greece	Apr-04	R. Bateman/PJR
O. exaltata	Trapani, Sicily, Italy	Apr-09	H. Breitkopf
O. ferrum-equinum	Marathea rd., S Pelepon., Greece	Apr-05	R. Bateman/PJR
O. fusca	Cilento, Campania, Italy	May-09	H. Breitkopf
O. garganica	Marina di Lesina, Gargano, Puglia, Italy	Apr-09	H. Breitkopf
O. heldreichii	E Hora Sfakion, Crete, Greece	Apr-04	R. Bateman/PJR
O. holoserica	Cilento, Campania, Italy	May-10	G. Scopece
O. incubacea	Cilento, Campania, Italy	Apr-10	H. Breitkopf
O. insectifera	Mti. Picentini, Campania, Italy	May-10	H. Breitkopf
O. iricolor	SW Kyrenia, NC, Greece	Mar-10	R. Bateman/PJR
O. levantina	Akrotiri, Cyprus, Greece	Feb-07	H. Paulus
O. lunulata	Passo delle Pontanelle, Sicily, Italy	Apr-10	H. Breitkopf

O. lutea	Pennina di Lupo, Sicily, Italy	Apr-09	H. Breitkopf
O. mammosa	Haifa, Israel	Mar-10	N. Vereecken/A. Dafni
O. morisii	Oristano, Sardegna, Italy	May-10	H. Breitkopf
O. oxyrrhynchos	Passo delle Pontanelle, Sicily, Italy	Apr-10	H. Breitkopf
O. pallida	La Ficuzza, PA, Sicily, Italy	Mar-10	R. Romolini
O. panormitana	SS121 - km140,2, CL, Sicily, Italy	Mar-10	R. Romolini
O. passionis	Larzac, France	May-10	R. Romolini
O. promontorii	Mte. St. Angelo, Gargano, Italy	Apr-10	R. Souche
O. provincialis	Saint Paul Enforet, France	Apr-10	R. Souche
O. reinholdii	Marathea rd, S Pelepon., Greece	Apr-05	R. Bateman/PJR
O. scolopax	NW LA Frayssinede, Guilhaumard, SC France	Jun-09	R. Bateman/PJR
O. sicula	Mte. Pellegrino, PA, Italia	Mar-10	R. Romolini
O. speculum	Sicily, Italy	Apr-09	H. Breitkopf
O. sphegodes	Capoiale, Gargano, Puglia, Italy	Mar-09	H. Breitkopf
O. tenthredinifera	Capoiale, Gargano, Puglia, Italy	Apr-09	H. Breitkopf
O. tetraloniae	Isernia, Italy	Jun-10	R. Romolini
O. umbilicata	Kephalos, Kos, Greece	Mar-02	H. Paulus
S. parviflora	Capoiale, Gargano, Puglia, Italy	Mar-11	H. Breitkopf

Fig.1a: Species in the phylogeny and two natural hybrids. 1—*Serapias parviflora*, 2—*O.speculum*, 3—*O.bombyliflora*, 4—*O.tenthredinifera*, 5—*O.sicula*, 6—*O.lutea*, 7—*O.fusca*, 8—*O.iricolor*, 9—*O.pallida*, 10—*O.insectifera*, 11—*O.aymoninii*, 12—*O.apifera*, 13—*O.heldreichii*, 14—*O.attica*, 15—*O.umbilicata*, 16—*O.levantina*, 17—*O.holoserica*, 18—*O.mammosa*, 19—*O.scolopax*, 20—*O.reinholdii*.

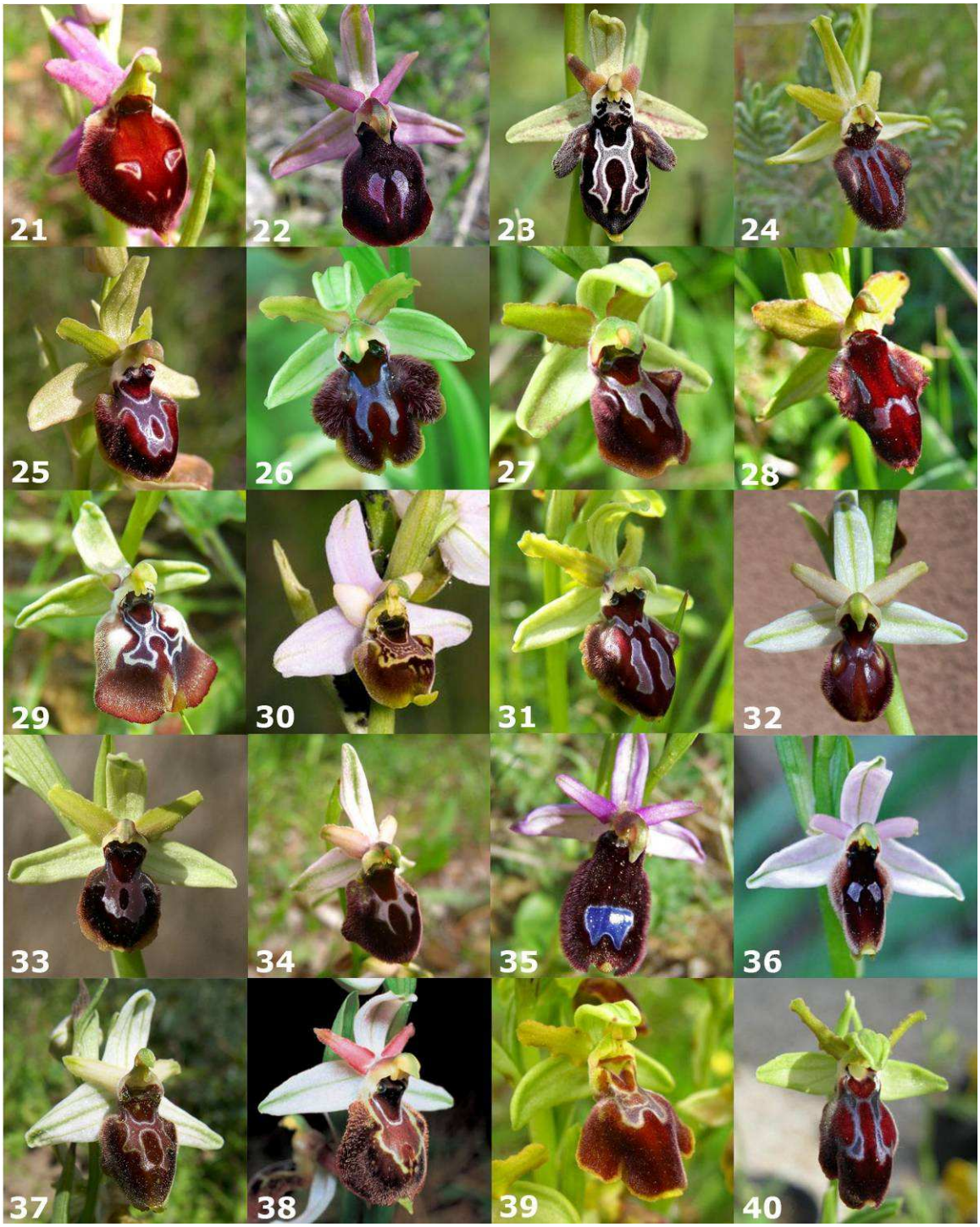


Fig.1b continued: Species in the phylogeny and two natural hybrids. 21—*O.crabronifera*, 22—*O.ferrum-equinum*, 23—*O.cretica*, 24—*O.incubacea*, 25—*O.provincialis*, 26—*O.panormitana*, 27—*O.sphegodes*, 28—*O.promontorii*, 29—*O.oxyrrhynchos*, 30—*O.tertraloniae*, 31—*O.garganica*, 32—*O.exaltata*, 33—*O.passionis*, 34—*O.morisii*, 35—*O.bertolonii*, 36—*O.lunulata*, 37—*O.archipelagi*, 38—*O.arachnitiformis*; natural hybrids: 39—*O.sicula x panattensis*, 40—*O.bilunulata x garganica*.

PCR & SEQUENCING

Newly designed and previously published primers were tested using polymerase chain reaction standard protocols and a 2720 Thermo Cycler (Applied Biosystems) with annealing temperatures ranging between 45°C and 63°C. With each primer couple at least three PCR's in a range of +- 5°C difference from the predicted melting temperatures were performed. Cycling conditions were as follows: 3 min initial denaturation at 94°C, followed by 35 cycles of 94°C 45 sec; TA 45 sec; 72°C 90 sec and a final extension of 72°C 7 min; 4°C hold. Annealing temperatures were 58°C for *ACS*, *FAD* and *MYB*, 59°C for *BGP*, 60°C for *LFY* and 65°C for *CAD*.

All PCR reactions were performed in a final volume of 25µl containing 50 – 100 ng DNA template, 10 pM of each, forward and reverse primer, 200µM of each dNTP, 2µl of 10x Taq buffer (50 mM KCl, 10 mM Tris-HCl pH 9), 0.5 µl 1.5mM MgCl2 and 0.5 U of Taq polymerase (Pharmacia, Amersham Biotech). PCR products were separated on a 1.5 % agarose gel stained with ethidium bromide (0.3 mg/l) and photographed under UV light using a Gel Doc 2000 system (Biorad). Only primers that yielded discrete bands were further investigated; those that yielded a smear or no amplification were discarded. Products with multiple bands were separated in a 1.5 % TBE agarose gel, excised and purified using a kit. Those products that gave single bands were purified using IllustraTM GFX PCR DNA Purification Kit (GE Healthcare). Quality and concentration of the purificates was checked with a NanoDropTM 1000 Spectrophotometer (ThermoScientific). Amplificates were further amplified using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc.) and purified following the ethanol-sodium acetate precipitation protocol provided with the kit. The amplification of the *LFY*-genes' first intron was done as described in Schlüter et al. (2007). Sequencing took place on 3130 and 3130xl Genetic Analyzers (Applied Biosystems, Inc.). Sequences were then analyzed with Sequence Analyzer 5.2 (Applied Biosystems, Inc.) and the chromatograms read out with Sequence Scanner 1.0 (Applied Biosystems, Inc.) or Chromas Pro (Technelysium Pty Ltd).

CLONING & PARALOGY ASSESSMENT

Heterozygosity and paralogy were expected to be issues when dealing with nuclear genes of the predominantly outcrossing genus Ophrys. If a sequence was partially unreadable due to existence of an indel in one of the two alleles, the PCR product was cloned into a bacterial vector (pGEM-T Easy, Promega) and inserted into competent *E. coli* cells by chemical transformation. Ambiguous SNP's were ignored as it was the aim to obtain consensus sequence. Cells were plated out on LB growth medium containing Ampicillin, allowing only the successfully transformed cells to grow colonies. Colonies were repicked to new plates for backup and storage. A small portion of the colonies was used as template for a PCR with the conditions following the manufacturer's protocol using the primer couple T7 / SP6 provided with the cloning kit (pGEM-T Easy, Promega). Sequencing was done as described above, but using the primers of the marker in question. Five to ten clones per accession were sequenced. If more than 2 alleles were detected in the cloning procedure the primers were identified to amplify at least one other copy of a gene/gene family and consecutively excluded. As *Ophrys* is generally diploid, no more than two alleles of any gene should be gathered from a single individual. The few species that have been shown to be tetraploid are not included in this study. Cloning was applied for unphasing hetereozygotes in *ACS*, *BGP*, *CAD* and *FAD*. For *LFY* we used the nested primers of Schlueter et al (2007) + three newly developed nested primers. *ITS* and *MYB* sequences were obtained by direct sequencing.

We detected a notable haplotype differentiation in some genes, i.e. the obtained haplotypes could be classified into two or more groups regarding sequence similarity. To rule out the possibility that our PCR primers amplify paralogs, additional to the cloning procedure differential primers for the two most prominent sequence types/haplotype groups were designed for two genes (*CAD*, *FAD6*) for amplification and sequencing. PCR conditions were as described before with an annealing temperature of 59°C. We tested seven species with the new primers. Again, no more than two alleles per species could be detected.

Tab.3. – Primers used in this study and their respective annealing temperatures. *= nested haplotype group specific primers for paralogy assessment. **= nested sequencing primers. All other primers can be used for both, PCR and sequencing

ACS-F	AGGTTGAGATTGCATTTGTGG	58°C
ACS-R	TTCAACAGCTTTTCTTTCATCG	
BGP-F	GCTGGCAACAATAGGATCTCCA	59°C
BGP-R	ACTGAGGAGCTTCCATCTAC	
CAD-F	CTACTTCTTCGGCGAGGCTAC	65°C
CAD-R	AGATGCTGTATGGAAGACACC	
CAD-1F*	TGCATTTTGAATTCTGTTACTTAT	59°C
CAD-1R*	AATCAGCTCTATACAAATGCA	
CAD-2F*	ACATTTTGGATTGTGTTACTT	59°C
CAD-2R*	AAATCAGCTCTAAACAAATGCAA	
FAD-F	ATATCACGCTCAGAGACATTATTACAAC	58°C
FAD-R	ATATGTCTTCCACCAACTTGTTCTTTG	
FAD-1F*	CTAGGCTTTGAACGTATCTCTTA	59°C
FAD-1R*	GACCTATGCGCACAATCATGA	
FAD-2F*	CTAGGCTTTGAACTTATCTCTTG	59°C
FAD-2R*	GACCTATGCGCACAATCATGG	
ITS-JK14	GGAGAAGTCGTAACAAGGTTTCCG	55°C
ITS-JK12	CCAAACAACCCGACTCGTAGACAGC	
LFY-E1Cf	ATGGTGCTGGCCACATCGCAGCAACA	60°C
LFY-E2Gr	GAAGAGGTAATCGAGCCCGTTCTTCTTAGCYC	
LFY-I1Hf**	ATCGAAACTTATGCATCTTCAGC	60°C
LFY-I1Sf**	TCATGTTTCAATCAGGCGCGAT	
LFY-I1Sr**	ATCGCGCCTGATTGAAACATGA	
MYB-F	GGAATTCCCTTGCTCTTTGTGC	58°C
MYB-R	GAGGTAATTGAGCCACCGAAGC	

SEQUENCE EDITING & ALIGNMENT

Sequences were assembled and aligned with the software BioEdit (Hall 2001) using the implemented ClustalW algorithm (Thompson et al. 1994). As computerized algorithms have been found to be inaccurate (Morrison 2009), the alignments have been checked by eye for apparent mistakes and manually

corrected to minimize homoplasy. Alleles from heterozygous individuals were merged into a consensus sequence using IUPAC coding. The alignment followed a two step procedure in which (1) all ingroup sequences were aligned to each other, followed by (2) alignment of the outgroup. Regions that could not be unambiguously aligned were discarded prior to phylogenetic analyses.

PHYLOGENETIC ANALYSIS

The combined dataset was analyzed with three different methods: MrBayes (Ronquist & Huelsenbeck 2003) for Bayesian inference, RAxML VI (Stamatakis 2006) for maximum likelihood, and PAUP* 4.0b10 (Swofford 2002) for parsimony reconstruction. As the single gene data sets yielded poorly resolved and supported trees, the matrices of all seven genes were combined into just one. As gaps are treated as missing data in all analyses, phylogenetic useful information from insertion and deletion events was coded as 0/1/- (absent/present/inapplicable) as described in Simmons and Ochoterena (2000) using the software GapCoder (Young & Healy 2003). The combined dataset had a length of 6275 characters: 5990 bp + 285 gap states. For Bayesian and parsimony analysis the best fitting model of molecular evolution for each gene was chosen using the Akaike information criterion (AIC) as given in the output of MrModelTest 2.3 (Nylander 2004). Selected models were: *ACS*: GTR, *BGP*: HKY+G, *CAD*: HKY+G, *FAD*: GTR+I, *ITS*: SYM+G, *LFY*: GTR+G, *MYB*: F81+I. RAxML has the GTR+CAT approximation implemented, which "represents an efficient computational work-around for the GTR+G model" (Stamatakis 2006b), which was proposed by the AIC for the combined dataset. *Serapias parviflora* was used as outgroup in all analyses.

Bayesian analysis was conducted on an external computer cluster (CBSU BioHPC, Cornell University) with 2 separate runs of four Markov-chain Monte Carlo (MCMC) chains for 10 million generations with tree sampling every 1000 generations. 25% of the sampled trees were discarded as burnin and of the remaining samples only the 25% best scoring trees were used to calculate the species tree and posterior probabilities. Runs reached convergence with standard deviation split frequencies below 0.01. ML analysis was computed with the software RAxML VI (Stamatakis 2006). Following the heuristic search and a run of 1000 bootstrap replicates the best-scoring ML-tree was identified.

For the parsimony analysis with PAUP* we conducted a heuristic search with the following settings: tree bisection reconnection (TBR) branch swapping, MulTrees, 'steepest decent' and 100 replicates of random sequence addition. Robustness of the topology was assessed with 1000 bootstrap replicates and the settings of the heuristic search, except the options MulTrees and 'steepest descent' turned off.
We analyzed different alignment types of the combined dataset under ML to assess their impact on tree topology and bootstrap support: (1) a matrix containing the complete combined dataset (without gap states), (2) a matrix with major ambiguous alignment positions excluded, and (3) a matrix with ambiguous positions and gaps excluded. Major ambiguous positions were defined as columns in the alignment containing ambiguity (IUPAC) codes in more than 5% of the sequences. As SNP's in heterozygous individuals are treated as missing data in respect to homozygotes, the exclusion of these positions from the phylogenetic analysis is likely to minimize bias.

DISTRIBUTION, MORPHOLOGY & ECOLOGY DATA

We plotted data from spatial distribution, floral morphology and pollination onto the tree topology to infer hypotheses about biogeography, floral trait evolution and pollination syndrome evolution in *Ophrys*. Various sources of literature were screened (e.g. Gaskett 2011, Van der Cingel 1995, Delforge 2006) to extract data for four categories of traits that are likely to be correlated to organismal history, and therefore the species tree topology: (1) for floral morphology we concentrated on petal color and shape. the coloration of the outer petals were categorized into four classes of colors/color combinations that have been observed to occur within a single species: green, red/white, green/red/white and green/white. A helmet-shaped median outer petal, as well as minute inner petals are generally found in only few species/species groups, and could therefore represent very ancestral or strongly derived floral traits. (2) Pollinators of the investigated *Ophrys* species can be assigned to a modest number of genera and families of bees and wasps. Bee pollinators: *Eucera*, *Melecta* and *Anthophora* (Anthophoridae); *Andrena* (Andrenidae); *Osmia* and *Chalicodoma* (Megachilidae); *Colletes* (Colletidae). Wasp pollinators: *Argogorytes* (Sphecetidae); *Dasyscolia* (Scoletidae). (3) After comparing patterns in *Ophrys* an assignment of species to four areas of distribution seemed feasible. Main distribution areas are: the West- and Central Mediterranean, the East-

Mediterranean, the whole Mediterranean, and Central Europe + West Mediterranean. (4) As the differential pollinia placement in different groups of *Ophrys* effects mechanical reproductive isolation, this trait was also chosen to be compared with the tree topology. The two mutually exclusive categories are: pollinia placement on the insects head, or the abdomen.

RESULTS

PHYLOGENETIC ANALYSIS & SPECIES TREE

The combined data set of 6275 characters: 5990 bp and 285 gap states. 1556 positions were variable, 648 of those potentially parsimony informative. The equally most parsimonious trees produced in the parsimony analysis had a length of 2249 steps, a consistency index (CI) of 0.74 and a retention index (RI) of 0.82. The single gene matrices were analyzed with Bayesian inference (Fig.2) and the respective models proposed by AIC. ML analyses under the GTR-CAT approximation yielded similar but generally less supported topologies (not shown). The genealogies have significantly different tree topologies, with a general trend to consensus branching in lower lineages and no consensus/resolution towards the terminals (Fig.3). Bayesian inference, parsimony and ML phylogenetic analyses of the combined data set resulted in similar topologies. As expected from the pattern observed by comparison of the genealogies, the resulting species tree has good support at lower levels in all three analysis types, but topology and support at higher levels of the tree are highly dissimilar. Bayesian inference produced the most resolved and supported species tree (Fig.2). Bayesian posterior probabilities (PP), parsimony bootstrap percentages (BP_P) and maximum likelihood bootstrap percentages (BP_{ML}) are given above branches.

Two strongly supported main lineages were discovered in all analyses. (A) a clade containing basally branching species of sect. Euophrys (*O. speculum, O. bombyliflora, O. tenthredinifera*) and a crown group of all included taxa of sect. Pseudophrys, where *O. lutea s.l.* (*O. sicula* + *O. lutea*) are sister to the remainder of the section. Not strongly supported in all analyses are the basal positions of *O. speculum* and *O. bombyliflora*, as well as the branching order of

the *O. fusca s.l.* taxa (*O. fusca, O. iricolor, O. pallida*). Though, all splits receive strong PP support. (B) a clade containing taxa of only sect. Euophrys. The three most basal splits have high support in all analyses: ((*O. insectifera* + *O. aymoninii*) (*O.apifera* (*O. heldreichii* (remainder on main clade 2)). Most of the higher splits receive strong PP support, but mostly very low bootstrap. Some species grouping have moderate to strong support in at least two analysis types: an East-Mediterranean *O. umbilicata* group (*O. umbilicata*, *O. attica, O. levantina*), and some groupings of East- and Central Mediterranean species. *O. sphegodes* + *O. promontorii*, *O. garganica* + *O. exaltata* + *O. passionis* + *O. morisii*, and *O. archipelagi* + *O. arachnitiformis*. The latter two ensembles form a strongly supported clade (except ML) together with *O. lunulata, O. bertolonii, O. tetraloniae*, and *O. oxyrrhynchos*, of which all are Central-Mediterranean distributed.

The accuracy of our approach to conduct a combined analysis of all alignment positions was reconciled by calculating trees under ML, with or without the retention of gaps and/or ambiguous alignment positions. The resulting trees topologies are in many parts congruent (Fig.4). As expected, the differential impact of the three different approaches is most obvious within higher level clades, in which genetic differentiation is generally low. Most striking is the positioning of two basally diverging taxa: While in the complete data set (tree 1) *O. bombyliflora* is included with weak support in main clade A, and the position of *O. speculum* regarding to the main lineages is unresolved, we find a different situation for the reduced data sets. Excluding major ambiguities (tree 2) leads to an inclusion of *O. speculum* in main clade A, which is even strongly supported when also gaps are excluded (tree 3). Similarly, the position of *O. bombyliflora* is unresolved in tree 2, but within tree 3 the taxon is included in main clade A, though with weak support.

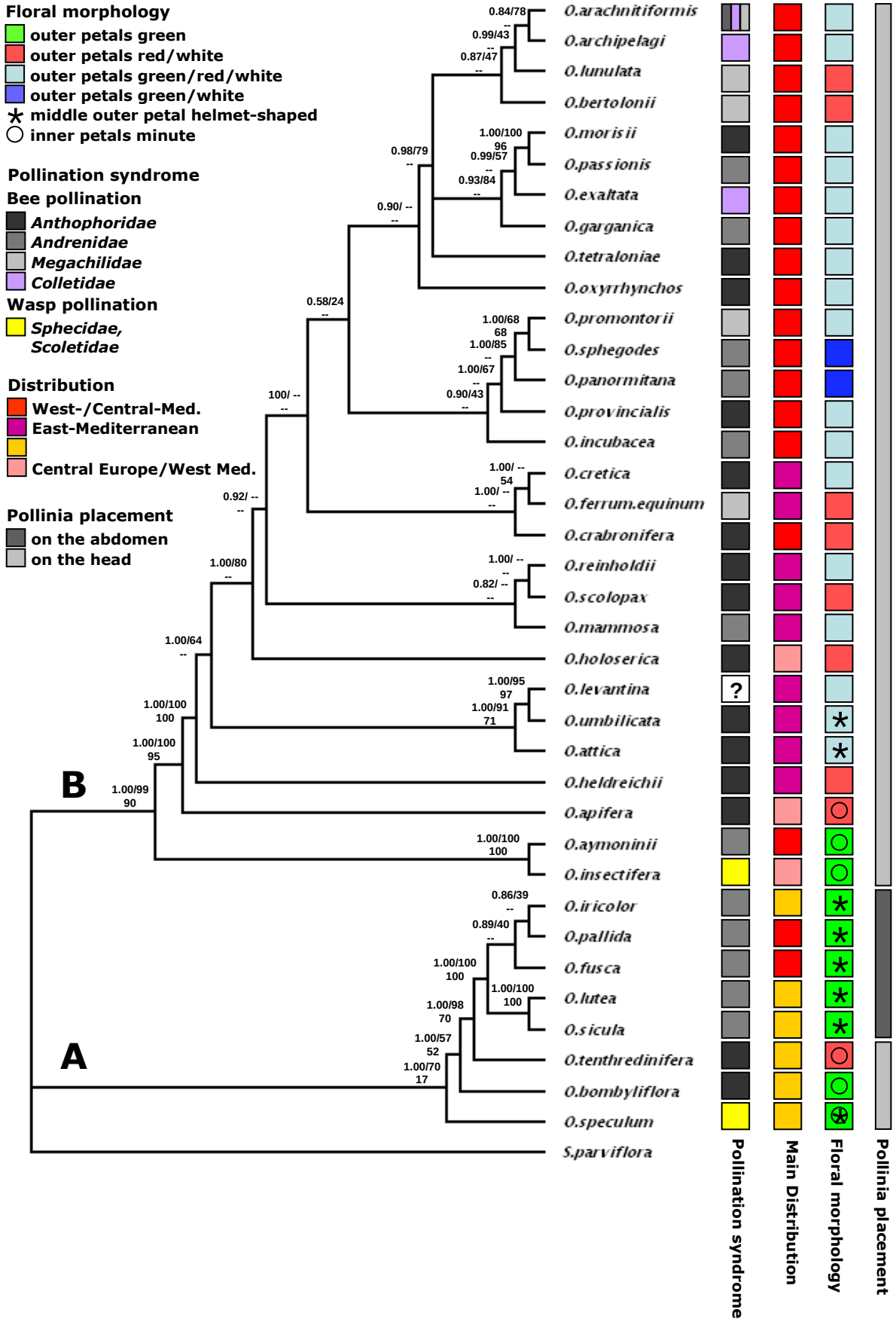
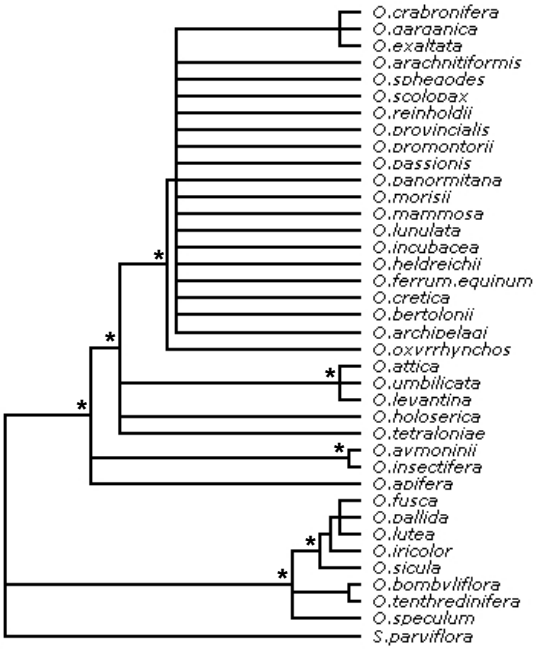
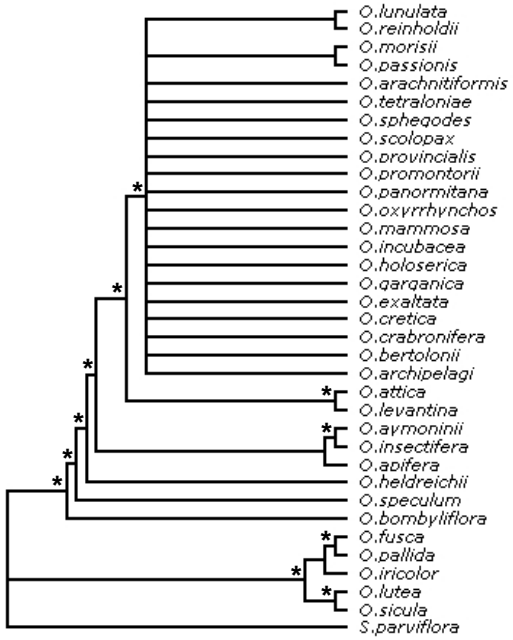


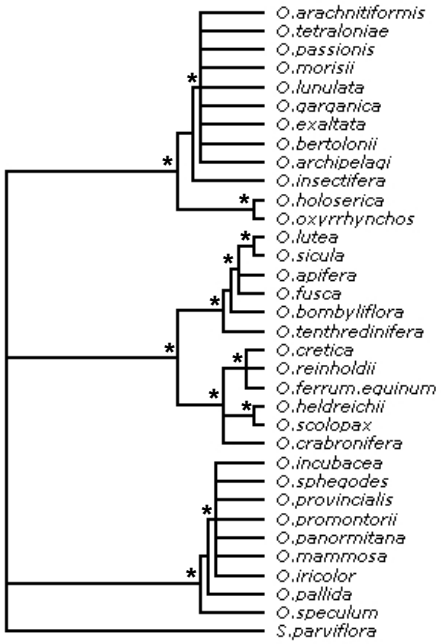
Fig.2 – *Ophrys* Species tree. Numbers above branches indicate PP (Bayes), MP (PAUP) and ML (RAxML) bootstrap values. The tree topology is that of the Bayesian analysis.



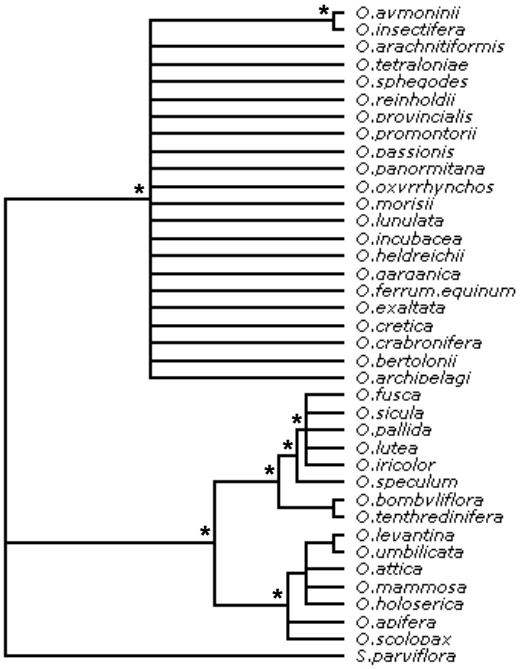
ACS



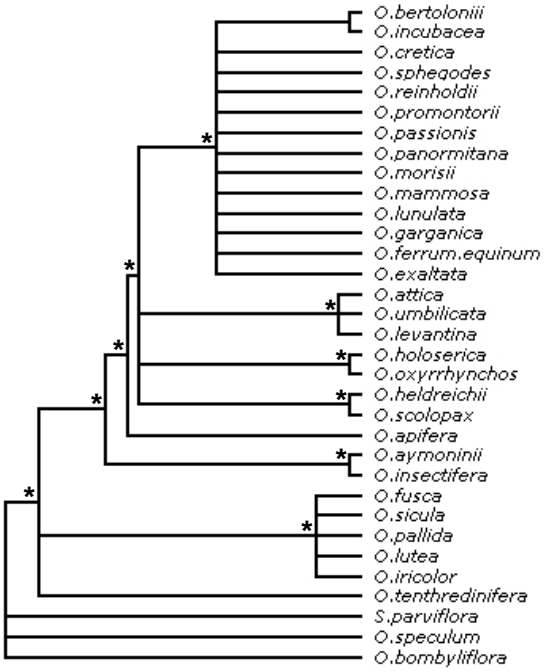
BGP



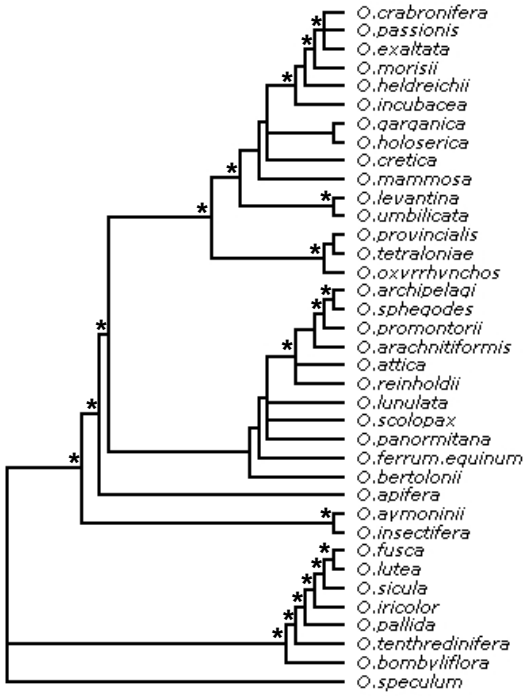
CAD



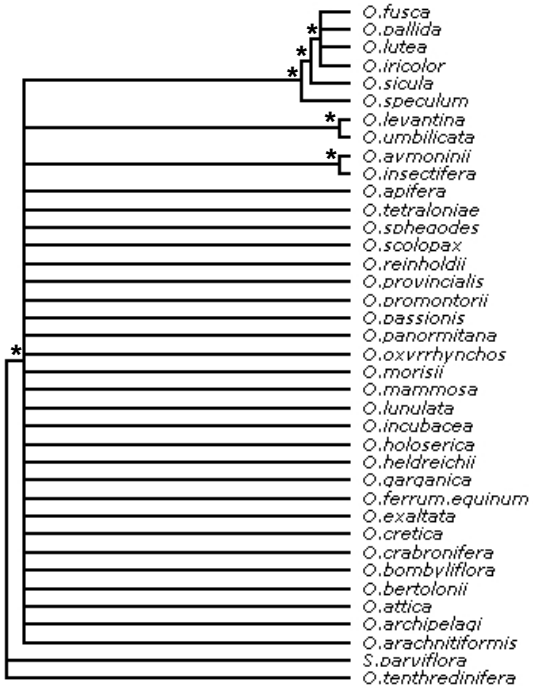
FAD



ITS

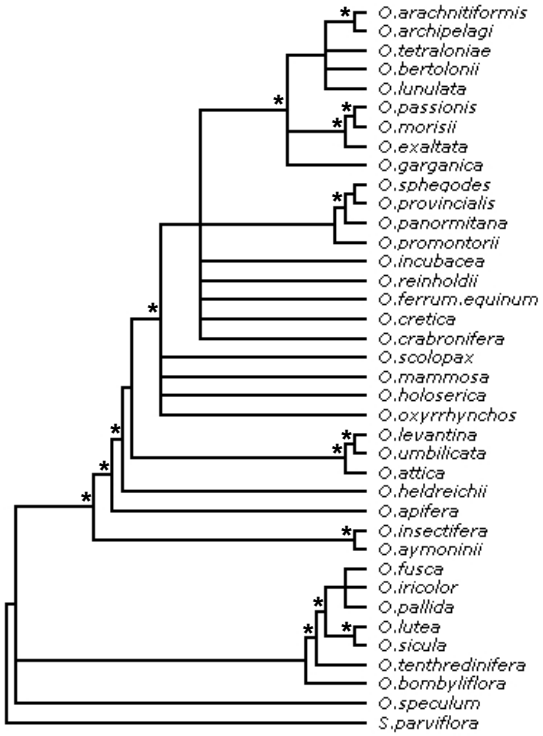


LFY



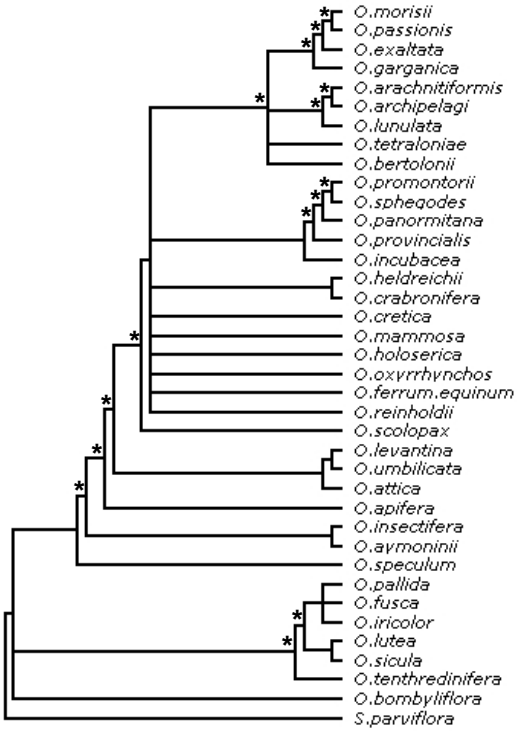
MYB

Fig.3 – Genealogies. Phylogenetic bayesian analyses with MrBayes and the models proposed by AIC. 2 runs with 4 chains on BioHPC cluster at Cornell University. 0.7 – 3.0 million generations runtime til convergence was reached. Branches with BPP < 0.50 collapsed, those with BPP > 0.75 marked with *. In some analyses one or few accessions are represented with a partial sequence or are missing completely: see Tab.X.

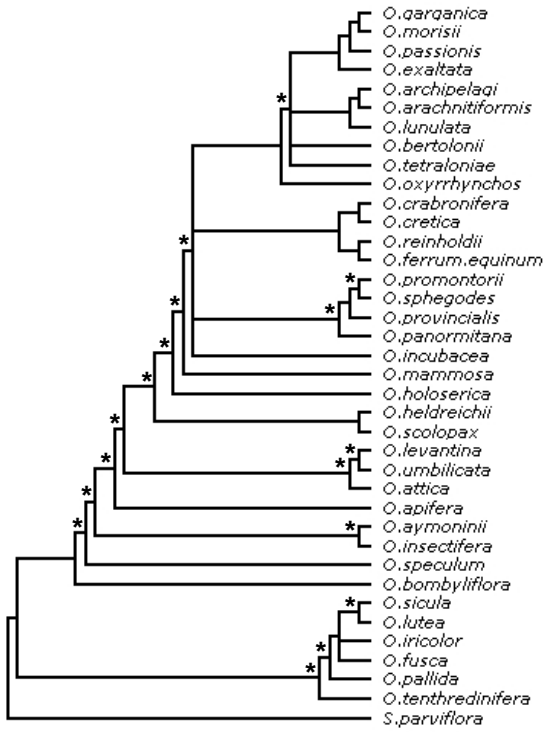


ML combined (1)

Fig.4 – ML Species trees to compare different alignment types. Phylogenetic maximum likelihood analyses with RAxML unter the GTR-CAT approximation and 1000 bootstrap replicates. Branches with BS < 75% collapsed, those with BS > 0.80 marked with *. In some gene matrices one or few accessions are represented with a partial sequence or are missing completely: see Tab.X. Trees are shown for the combined analysis of (1) all genes + gap states matrix (left), for (2) all genes excluding major ambiguous positions* (lower left) and for (3)all genes excluding major ambiguous and indel positions. * = more than 5%. i.e. 2 sequences of 38 overall.



ML combined (2)



ML combined (3)

DISKUSSION

OPHRYS MULTI-LOCUS NUCLEAR GENE PHYLOGENY

The combined analysis of sequences from multiple nuclear genes seems an adequate approach to infer a species tree rather than a gene tree. This is even more important in a presumably recently radiating plant group, in which gene histories are more likely to be obscured by processes of deep coalescence and/or introgression (Maddison 2006). The achievement of the phylogenetic relationships between the major species groups allows us to infer biogeographic history and pollination system evolution in this intriguing plant group.

PREVIOUS MOLECULAR PHYLOGENETIC STUDIES IN *OPHRYS*

Due to the presumably high degree of homoplasy in morphological characters, taxonomic treatments employing a phenetic approach differ substantially. They are therefore not further discussed here. Wide acceptance only found a division of the genus in two sections (or subgenera): while in sect. Ophrys (or: Euophrys) the pollinia are attached to the pollinators head they are attached to the abdomen in sect. Pseudophrys; the differential pollinia placement is dictated by sets of morphological characters exclusive to the two sections. Arguably most important of those is the direction of hairs on the flower labellum.

Bateman (2003) focussed on the phylogeny of all Orchidinae, including a representative set of 32 *Ophrys* species, but used only *ITS*. Soliva found no significant incongruence in the phylogenetic signals of *ITS* and *trn*L-*trn*F for 18 species and combined the datasets into one matrix, while Devey (2008) obtained differing topologies in the trees of *ITS* and *trn*H-*psb*A/*trn*D-*trn*T for 85 accessions. The latter study revealed ten well supported species groups: *O. insectifera, O. tenthredinifera, O. speculum, O. bombyliflora, O. fusca, O. apifera, O. sphegodes, O. fuciflora* (syn. *O. holoserica*), *O. scolopax* and *O. umbilicata*. The position of *O. apifera* as sister to *O. sphegodes* + *O. fuciflora* + *O. scolopax* + *O. umbilicata* is supported by *ITS* and *ITS* + *trn*L-*trn*F, but not by *trn*H-*psb*A + *trn*D-*trn*T. *O. tenthredinifera*, *O. speculum* and *O. bombyliflora* are always placed between the root of the tree and the *O. fusca* group, though unresolved or in different order with low support. *O. insectifera* is placed as sister to all other *Ophrys* (*ITS*, Devey 2008), to the large sect. Ophrys clade, or to *O. tenthredinifera* + *O. speculum* +

O. bombyliflora + *O. fusca*. Devey cloned accessions found to be heterozygous in *ITS*. Interestingly, different copies of the unphased *ITS* often group in very distant clades. Also some homozygous accessions are placed in groups or even sections that are well different from the phenotype of the accessions (e.g. *O. balearica* within *the O. scolopax* group, *O. dyris* in the *O. holoserica* group).

OUR SPECIES TREE TOPOLOGY

In contrast to previous studies we achieved resolution and robust support for the branches of all basal lineages up to the *O. umbilicata* group. We found a strongly supported division of the genus into two main lineages with *O. insectifera* and *O. speculum* as basal branching species of the clades. This is in accordance to the findings of Soliva (2001), although the position of *O. insectifera* was weakly supported. Instead, *ITS* only places this taxon as sister to all other species (Bateman 1997, Devey 2008) with only moderate support. Accordingly to previous studies we found a monophyletic sect. Pseudophrys nested within members of sect. Euophrys, the latter one being paraphyletic. In main clade A, *O. speculum* in the basal position is hierarchically followed by *O. bombyliflora, O. tenthredinifera*, and the *O. fusca* group (= sect. Pseudophrys), in which *O.sicula* + *O. lutea* are sister to *O. fusca* + (*O. pallida* + *O. iricolor*). In main clade B, *O. insectifera*, is followed by *O. apifera*, *O. heldreichii*, and the *O. umbilicata* group, which is sister to a crown group of taxa from the *O. sphegodes*/*O. holoserica*/*O. scolopax* groups. Resolution in this part of the tree is often only supported by the Bayesian and the parsimony analysis. It can be assumed that the latter groups are young compared to other lineages in *Ophrys*. Dating of *Ophrys* and some of its major lineages should be possible (Gustafsson 2010) and would extend our knowledge of the timing of evolutionary processes such as lineage sorting and the emergence of genetically distinct lineages.

POLLINATION SYNDROME EVOLUTION

The selective pressures imposed by the pollinator species is directly correlated to its spatial and temporal occurrence. Strength and direction of selective pressures are expected to be more dynamic than in unspecialized pollination systems, as the sensitivity of the pollinating insects to environmental and climate change adds to that of the plants. Furthermore, alkene production in orchid flowers is a

pre-adaptation for the evolution of sexual deception and the attraction of male bees as pollinators, which in turn offers numerous chances for divergent selection in the genus *Ophrys* (Schiestl & Cozzolino 2008). For long time *O. speculum* and *O. insectifera* were the only known wasp-pollinated *Ophrys* species. They are pollinated by *Argogorytes mystaceus*/*combinata* (Sphecidae) and *Dasyscolia ciliata* (Scoliidae), respectively. But *O. cilicica*, a close relative of *O. cretica*, has been found to be pollinated by *Argogorytes sp*. This also explains the morphological convergence to *O. insectifera*, which has the same wasp pollinator. As with *O. speculum* and *O. insectifera* at the basis of the two main lineages (see Fig.2), we confer that wasp pollination is the ancestral pollination syndrome in *Ophrys*. Bee pollination has evolved two times independently with at least one return to wasp-pollination (*O. cilicica*). On the basis of Malyshev (1968) and Michener's (1974) notation, that the two wasp groups in question have evolved earlier than the Apoidea, and the fact that adaptive evolutionary processes shaped *Ophrys* pollination syndromes, Kullenberg and Bergström proposed already in 1976 the wasp pollinated taxa to be older than bee pollinated ones. When we apply this rationale to the observed pattern, that most basal taxa (i.e. *O. bombyliflora, O. tenthredinifera, O. apifera, O. heldreichii, O. umbilicata* groups) are pollinated by bees of the genus *Eucera* (Anthophoridae) (Fig.2), it can be assumed that pollination by *Eucera* bees has evolved prior to other bee pollination syndromes in this clade, and that *Eucera* bees might have appeared/radiated earlier than for example the genus *Andrena*, of which many members are known to pollinate *Ophrys* species of the highly diverse *O. sphegodes* and *O. fusca* crown groups. As those two groups are species-rich and attract a large number of different *Andrena* species, it can be assumed that the radiation of the *Andrena* bees is positively correlated to the diversification of the *O. sphegodes* and *O. fusca* groups. A survey of reported pollinators for the great part of described taxa in *Ophrys* showed that the *O. umbilicata-holoserica-scolopax* groups are mainly *Eucera* pollinated (98%); the *O. fusca-lutea* groups are mainly *Andrena* pollinated (86%) with some switches to *Anthophora, Chalicodoma* and *Colletes* in the *O. dyris/atlantica* sub-groups; pollination syndromes diversified notably in the *O. sphegodes* group, with *Andrena* (44%), *Chalicodoma* (18%), *Anthophora* (15%), *Osmia* (7%), *Melecta* (6%), *Colletes* (6%), and *Xylocopa* (4%).

BIOGEOGRAPHY / RADIATION

The genus is principally Mediterranean distributed, with a handful of species in Central Europe and only one species each in Southern Scandinavia (*O. insectifera*) and the Canaries (*O. bombyliflora*). Centres of diversity are located in the Central and Eastern Mediterranean basin (Nelson 1962), where the species groups around *O. fusca, O. speculum, O. tenthredinifera, O. holoserica and O. scolopax* diversified notably. Plotting distribution data onto the *Ophrys* phylogeny, an obvious pattern emerges: (1) widely distributed taxa (all-Mediterranean / Central-European and Mediterranean) are generally more ancestral species of the genus, (2) all-Mediterranean distributed taxa can only be found in main clade A, there in ancestral positions, (3) Central-European distributed taxa can only be found in clade B, in mostly ancestral positions, (4) East Mediterranean taxa cluster at the base of clade B, (5) West- and Central-Mediterranean taxa cluster as crown group of clade B. The following scenario could explain the distribution pattern: Older species of the genus had sufficient time to spread over large areas of the European continent, as the key innovation of sexual deception allowed them to enter an open ecological niche. Species then locally adapted to novel pollinators, evolving new species. Following the colonisation of the Mediterranean, the genus underwent radiations that are likely correlated to the disclosure of new groups of bee pollinators. Ancestral members of the mainly *Eucera*-pollinated *O. umbilicata*, *O. scolopax* and the mainly *Andrena*-pollinated *O. fusca* groups diversified in the East-Mediterranean basin with ancestors of *O. heldreichii* and the *O. umbilicata* group as central figures of early divergence, while later in history the mainly *Andrena*-pollinated *O. sphegodes* group diversified in the Central- and West-Mediterranean basin. We conclude that at least two major radiation events in different parts of the Mediterranean contributed to *Ophrys* diversification. In the recent history of *Ophrys*, larger colonisations through the local variants were prevented as ecological niches are already occupied by other *Ophrys* species with the same pollinators. Contact of two distinct species with pollinator sharing will likely lead to hybridization, ending up with the absorption of the introgressing species. Replacement of the introgressed species through the introgressant is less likely due to the presumably smaller effective population size of the latter.

FLORAL MORPHOLOGY

The correct reconstruction of ancestral morphological character states is hindered by the occurrence of homoplasy due to repeated adaptation of different lineages to the same pollinator. Nevertheless, correlating some floral characteristics to the tree topology gives rise to the hypothesis that their occurrence is linked to organismal history. Green (i.e. non-colored) outer petals, mostly in combination with minute inner petals only exist in the ancestral lineages of both main clades and the *O. fusca* group. A helmet-shaped median outer petal characterizes *O. speculum* and all members of the *O. fusca* group. This structure is absent from members of main clade B, with the notable exception of *O. attica and O. umbilicata.* The finding of an *O. fusca*-like allele in the *LFY* gene of *O. umbilicata* corroborates the hypothesis that this taxon is of hybrid origin or heavily introgressed by a member of the *O. fusca* group. Colored petals are found in *O. tenthredinifera* from clade A and in all members of clade B, except for the basal branching *O. insectifera*. Species with the same petal colors or color combinations tend to cluster together (e.g. *O. sphegodes* and *O. panormitana*) but this trend is not significant over the species ensemble of clade B. Two small light-reflecting round structures close to the stigmatic cavity, called pseudo-eyes, characterise all *Ophrys* except the nested *O. bombyliflora* and the *O. fusca* group, in which they are absent as the result of secondary loss. Green outer petals, minute inner petals, pseudo-eyes, and probably also helmet-shaped median outer petals represent ancestral morphological character states in *Ophrys*.

HYBRIDIZATION VS. ISOLATION IN *OPHRYS*

Species delimitation presents a controversial discussed issue of biology in general, with *Ophrys* being a prime example for the problems associated with finding an appropriate species concept. The aim is to define a fuzzy-boarded entity, part of a continuum from individuals, populations, over varieties and subspecies, to reproductively perfect isolated species. When speaking about species boarders, hybridization has to be considered. The relative contribution of hybridization to the evolution of *Ophrys* is yet unknown. Though, a deeper knowledge regarding this aspect is needed to inform the decision of taxonomists and scientists, where to set species boundaries.

Many properties of *Ophrys* species make hybridization a likely scenario. First of all, *Ophrys* species are inter-fertile. All isolation barriers acting in sympatry have been shown to be leaky (Cortis 2008, Xu 2011). Then, many species have more than only one pollinator, which increases the likelihood of pollinator sharing in sympatry. Also for species that have been thought to attract only one species, additional pollinators have been observed. Besides the main pollinator(s), other insects are visiting *Ophrys* flowers. In an investigation of the pollinator spectrum for a single species from the Tyrrhenian Coast of Italy (Breitkopf, unpubl.), we repeatedly found five different visitor species: the bees *Andrena bimaculata*, *Andrena nigroaenea*, *Melecta sp*., *Xylocopa violacea* and a beetle, *Oxythyrea funesta* (Fig.5b). While *A. bimaculata* was identified as the main pollinator (Fig.5a), and *A. nigroaenea* as side pollinator, *Melecta sp.* was observed to attempt mating with the flower, though without pollinia removal. *X. violacea* was rarely landing on flowers (Fig.5b), also without pollinia removal. The beetle species has repeatedly been observed to use *Ophrys* flowers as a place to meet conspecific mating partners, and occasionally to take up pollinia (Figs. 5b/c). Also in a population of species with strong floral isolation at the Adriatic Coast of Italy (Xu, 2011), a few pollinators were caught while copulating on the 'wrong' *Ophrys* species (Schiestl, unpubl.). Taken together, these results show floral isolation to be leaky, at least in the investigated populations, and it indicates the presence of gene-flow, though without breaking down species boundaries.

Soliva (2003) demonstrated the existence of gene-flow between closely related *Ophrys* species with micro-satellites. Devey (2008) found allele sharing in *ITS* between species of more closely, but also far related groups, and took this as evidence for more recent hybridization, as *ITS* is known to have fast lineage sorting, i.e. quick homogenisation of different alleles compared to cp- and nrDNA. In the same study 165 neutral AFLP markers were scored for a representative set of species. The pattern observed is nearly congruent with that of the phylogeny presented here: species groups of clade A, and the *O. apifera* and *O. umbilicata* groups could be discriminated. All later branching species don't show a clustering conform with taxonomical treatments or the species groups discovered in *ITS*; i.e. species from different groups are intermixed in our nuclear gene tree and the AFLP's. In this light, it seems unreasonable that adding more markers to our dataset would have had changed the observed pattern.

We found allele sharing between distantly related taxa in *CAD*, where *O. apifera* and *O. fusca* have the same haplotype, and *O. iricolor* is very similar to members

of the *O. sphegodes* group. Haplotype sharing between members of the *O. sphegodes*, *O. holoserica* and *O. scolopax* groups is frequent in *ACS*, *BGP*, *FAD* and *LFY*.

Fig.5.a – Pollination of *O. sphegodes s.l.* from Cuma … through *Andrena bimaculata*

Fig.5.b – Flower visitors of *O. sphegodes s.l.* from Cuma. *Melecta sp.* (left), *Xylocopa violacea* (middle) and *Oxythyrea funesta* (right).

Fig.5.c – Pollination of *O. archipelagi* by *Colletes cunicularius* (left), *O. bilunulata* by *Andrena bimaculata* (middle); on the right: mating *Oxythyrea funesta* beetles on *O. sphegodes* (Moliterno, Basilikata, It.).

UTILITY OF NUCLEAR MARKERS FOR RADIATION PHYLOGENETICS

Nuclear genes are not only characterized by an overall higher rate of recombination and sequence evolution, but also by their huge range in evolutionary rates compared to organellar genomes. A relatively large effort in terms of time and money is needed to develop, test and select genes with evolutionary rates sufficiently high to identify recently diverging lineages. In such groups fast evolving genes are expected to yield different tree topologies. The addition of more markers is thought to increase the likelihood to obtain a species tree.

Our phylogeny profoundly increased our knowledge about the early stages of Ophrys diversification; the backbone of the tree is well resolved and strongly supported. The clades containing taxa from the *O. sphegodes*, *O. holoserica* and *O. scolopax* species groups are largely unresolved and/or exhibit some unexpected and questionable splits and groupings. This pattern might reflect gene flow between spatially closely distributed taxa, as indicated by the presence of a West- and Central-Mediterranean distributed crown group comprising taxa from different species groups. But this pattern is not consistent over the unresolved part of the tree.

We demonstrated the potential of a combined nuclear gene approach to elucidate familiar relationships in a group of plants, where organellar markers alone failed. Though, branching in the most complex groups could not be resolved with confidence. Also AFLP's based on 165 markers (Devey 2008) could not distinguish between the three most recently radiated species groups. Under this light the addition of just a few other nuclear loci to the dataset will probably not substantially increase the resolution potential. More likely a next generation sequencing approach with hundreds of markers is needed for a more fine-scaled species group definition in *Ophrys*.

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"ANALYSIS OF VARIATION AND SPECIATION IN THE *OPHRYS SPHEGODES* SPECIES COMPLEX"

INTRODUCTION

Studying evolutionary relationships in rapidly radiating complexes of closely related plant species presents a major topic in biology, and has its own technical and methodological challenges (e.g. Maddison 1997). The advantages of organellar DNA (haploidy, copy number, uni-parental inheritance) also hinder its use for studying speciation (Sota & Sasabe 2006). The use of diploid nuclear gene data is thought to overcome the shortcomings of organellar gene genealogies, as nuclear genes have been reported to evolve up to five times faster (Wolfe 1989, Ossowski 2010). The nuclear genome contains a vast amount of historical demographic information due to its large size and its bi-parental inheritance mode, which allows us to detect hybridization. Nevertheless, disadvantages in the use of nuclear genes stem from their frequent occurrence in gene families (paralogy), recombination, and a general lack of available markers for non-model organisms (e.g. Doyle 1997, Posada & Crandall 2002). The analysis of recent splitting events is further complicated through the frequent existence of retained ancestral polymorphism. The alleles of a heterozygous individual must not necessarily be reciprocally monophyletic, i.e. most closely related to each other in a collection of alleles. Coalescent theory predicts that the noise produced by incomplete lineage sorting can be reduced by sampling multiple alleles per species (Edwards & Beerli, 2000). But it also determines that genealogies will differ in their topologies, because the unlinked nuclear genes are differentially strong affected by introgression and recombination. Analysing multiple genes and alleles per species increases the probability, to approximate the underlying species tree, supported by the majority of the data (Small et al. 2004). Another way to analyse the evolutionary history of closely related species is the application of population-genetic, model-based approaches to infer the number and composition of clusters in a given sample of alleles (Pritchard et al. 2000, Huelsenbeck et al. 2011). Though un-rooted, they provide information about genetic distance and admixture.

The issue of species definition is a long-standing topic in biology and has been sprightly debated ever since (Mayr 1957, Hey 2001). Though, it has become

increasingly clear that all the different various species concepts can only be adopted for some organismal groups or taxa, but they are not universally applicable (Mallet 2007). Especially in plants, a lot of different processes and their potential complex interactions contribute to reproductive isolation (Widmer et al. 2008), which is the key attribute of 'good' species. This makes it difficult to rule out species boarders between incipiently diverging lineages.

The appraisal of results from studies at the border between species- and population-genetics often can yield hypotheses that conflict with existing classifications, invoking a re-consideration of the inherent species concepts.

The Mediterranean orchid genus *Ophrys* has not only attracted the interest of taxonomists and botanists ever since (e.g. Darwin 1862, Kullenberg 1961). It has become a (non-) model-system to study speciation and reproductive isolation. The genus can be split in a large number of lineages that are at least locally and temporally reproductively isolated enough to establish some morphological differences (Delforge 2006). As post-zygotic barriers are absent, reproductive isolation in sympatry is near exclusively based on floral isolation through specific male pollinators that are lured by the floral scent, a copy of the sexual pheromone of con-specific females, to repeatedly copulate on flowers of only a single *Ophrys* species, and bring by cross-pollination (Kullenberg 1961). But gene-flow, from slight introgression, over single hybrids, up to total admixture and hybrid speciation?, has been demonstrated (Soliva & Widmer 2003, Cortis et al. 2008, Souche 2008). Additionally to that, the dependence of *Ophrys* taxa on their specific pollinators, who's occurrence in turn is correlated to instable factors as vegetation succession, land-use and climate changes, is likely to be afflicted by strong fluctuations in the composition of the pollinator community. The species richness of the terminal clades is likely to be the result of a recent radiation that is characterized by dynamic speciation processes due to repeated pollinator shifts. An accelerated diversification rate in terminal clades could be explained by the exploitation of a novel, species-rich and diverse group of pollinators. Differences in sexual pheromone composition among closely related possible pollinator species are expected to be less strong developed than among well differentiated lineages.

Therefore, the adoption of pollinators from diversified bee genera as *Eucera* and *Andrena* might have triggered and facilitated a radiation of the plants. Molecular data (Devey et al. 2008) support at least three groups that presumably radiated recently: (1) a mainly *Andrena* pollinated *O. fusca* group, (2) an *Eucera*

pollinated ensemble of the *O. holoserica/scolopax/umbilicata* groups, and (3) a *O. sphegodes* group, in which a number of different bee genera function as pollinators. The latter group constitutes the most complex group due to its richness in species numbers and different pollination syndromes.

Under this light, the *O. sphegodes* complex represents an ideal study group for the application of more sophisticated model- and coalescent theory based approaches to infer attributes of demographic history from allelic data. The investigated taxa are an eligible group to study the utility of fast evolving nuclear genes, as traditionally employed organellar markers have failed to exhibit any sequence variability. Though, multi-locus analyses of un-phased nuclear gene data have been conducted on various groups of closely related animals (e.g. Sota & Sasabe 2006, Belfiore et al. 2008, Brumfield et al. 2008), examples from plants are still the rare exception (e.g. Städler et al. 2005, Arunyawat 2007). We therefore apply the outlined approach to a collection of alleles sampled from seven moderate to highly diverse nuclear genes of 19 diploid individuals that, based of different species concepts could be assigned just one strict bio-species, or 4 inclusive morpho-species (our concept), or as much as 14 eco-species.

We compare the usefulness of different species concepts in the light of our results, and then employ a phylogenetic approach to species delimitation in the O. sphegodes group.

MATERIALS & METHODS

For the present study, following an initial exploration of over 100 published and newly designed primer combinations, a set of nine nuclear gene markers was assembled and applied to sequence analysis of 19 accessions of 15 putative species of *Ophrys*, that can be assigned to four species groups from the conclusive *O. sphegodes* group, previously recognized by *ITS* analysis (Devey 2008). Notably, all cpDNA, and most nrDNA markers tested showed no sequence variation among closely related *Ophrys* species. Sequences were generated for the *PISTILLATA/GLOBOSA* locus (Cantone et al. 2008) and the eight newly developed nuclear gene markers (*ACS, BGP, CAD, FAD6, GPX, MYB, OMT*). Besides *MYB*, that is included as an example for the majority of investigated nuclear gene markers that showed no sequence variation between species of the complex *O. sphegodes* aggregate, all markers exhibit sequence variation

justifying to further investigate them. All markers had moderate to high levels of heterozygosity. Unphased sequence data was obtained by cloning individuals that were shown to be heterozygous in the direct sequencing.
We conduct basic population genetic analyses on these fast evolving nuclear genes, and try to infer attributes of demographic history by the application of coalescent and model based approaches. Informations on the investigated loci and the employed primers are given in Tabs.1 and 3.

PRIMER DESIGN, HOMOLOGY ASSESSMENT & MARKER EVALUATION

An EST-library from floral cDNA's of *Ophrys sphegodes* has been built up by colleagues of the Dept. of Systematic Botany (University of Zürich, Switzerland). The EST's were assembled automatically with an alignment software; the length of the gathered sequences varied from ~100bp to 1800bp. BLAST searches in public databases (e.g. NCBI, TAIR) allowed in many cases an ascertainment of the genes identity or an approximation in the sense of high sequence similarity to known genes. Sequences of genes that have been useful, i.e. phylogenetically informative in other studies, were chosen preferentially as candidates for primer design. The EST's contained only coding, exonic sequence. To assess the intron-exon structure and identify highly conserved regions for primer design, the EST's have been aligned with BioEdit Vers.7.0.9.0 (Hall, 2001) to that single, or in most cases multiple genomic sequences that were available in online databases, and showed the highest similarity (e-Value) with the EST in question. Where possible, genomic sequences from more closely related taxa (orchids, rice, asparagus) were included in the alignment. Primers have been designed in the exonic regions flanking one or more introns, depending on their size predicted by the alignment. As intron size varies notably within taxa from different families and genera, the gathered amplicon size often deviated largely from the prediction. To facilitate later applications an amplicon size of 300-900bp length was aspired. Primer design was done manually or using the online software 'Primer3' Vers.0.4.0 (Rozen & Skaletsky, 2000). Primers were checked for their expected annealing temperatures, hairpins and loops with 'OligoCalc' (Kibbe, 2007). Amplification was tested for different annealing temperatures. Primers that amplified multiple products were discarded. Single band PCR products were sequenced and compared to published sequences in gene bank. After confirmation that the primers amplified the gene they were designed for,

sequence variability and cross amplification was checked for closely, and also more distantly related taxa. To assess exon/intron structure and proof homology of the PCR products, new sequences were aligned with those used for the primer design. Lists of the markers selected for this study and their primer sequences are given in Tab.1. and Tab.3.

Tab.1 – Markers used in this study. Supposed gene function, length and primer annealing sites in the exons, rarely introns of the *A. thaliana* reference sequence.

Gene	Length aligned [bp]	Name / function of sequenced gene	Primer position in exon
ACS	671	Long-chain acyl-CoA-synthase-like	E6/E8
BGP	976	Beta-galactosidase-like	E14/E17
CAD	282	Cynnamyl alcohol dehydrogenase	E1/E3
FAD	453	Fatty acid desaturase	E3/E5
GGPS	177	Geranyl-geranyl-diphosphate-synthase	I?/E?
GPX	1025	Glutathione peroxydase	I4/E5
MYB	144	Myb transcription factor-like	E1/E2
OMT	706	O-methyltransferase-2-like	E1/E2
OrcPI	1005	*PISTILLATA*/*GLOBOSA*	E1/E5

SAMPLING & DNA-EXTRACTION

Fresh leaf tissue or flowers have been collected in the field, and either directly stored in silica gel, or fresh at -20°C. A list with all species accessions, sampling locations and collectors is given in Tab.2, while pictures of the sampled taxa are displayed in Fig.1.a-d. Fig.2 is a map with the sampling locations. DNA was extracted with a commercially available kit (GenElute Plant Genomic DNA miniprep kit, Sigma) or, for higher yields of DNA with a modified CTAB extraction method (Doyle & Doyle, 1990).

Fig.1.a – *O. incubacea* group (INC). f.l.t.r.: *O. incubacea* (GAR), *O. incubacea* (SAR), *O. incubacea* (CA), *O. promontorii*, *O. sipontensis*.

Fig.1.b – *O. exaltata* group (EXA). f.l.t.r.: *O. arachnitiformis*, *O. archipelagi*, *O. exaltata s.str.*, *O. splendida*, *O. tyrrhena*.

Fig.1.c – *O. sphegodes* group (SPH). f.l.t.r.: *O. argentaria*, *O. classica*, *O. sphegodes* (CA), *O. sphegodes* (GAR), *O. tarquinia*.

Fig.1.d – *O. garganica* group (GAR). f.l.t.r.: *O. garganica* (GAR), *O. garganica* (SIC), *O. garganica* (TUS), *O. passionis*.

Tab.2 – *O. sphegodes* group accessions used for this study, with sampling site, date, and respective collector.

Species	Sampling site	Date of collection	Collector
O. apifera (Outgroup)	Cilento, Campania, Italy	Jun-09	H. Breitkopf
O. arachnitiformis	France, exact loc. unknown	Apr-03	N. Vereecken
O. archipelagi	Capoiale, Gargano, Puglia, Italy	Mar-09	H. Breitkopf
O. argentaria	Caldine-Fiesole, Tuscany, Italy	Apr-09	H. Breitkopf
O. classica	Porto San Stefano, Tuscany, Italy	Mar-10	R. Souche
O. exaltata	Trapani, Sicily, Italy	Apr-09	H. Breitkopf
O. garganica (GAR)	Marina di Lesina, Gargano, Puglia, Italy	Apr-09	H. Breitkopf
O. garganica (SIC)	Taormina, Sicily, Italy	Mar-09	H. Breitkopf
O. garganica (TUS)	Alberese, Tuscany, Italy	Apr-09	H. Breitkopf
O. incubacea (GAR)	Marina di Lesina, Gargano, Puglia, Italy	Apr-10	H. Breitkopf
O. incubacea (SAR)	Laconi, Sardegna, Italy	Apr-10	H. Breitkopf
O. incubacea (CA)	Cilento, Campania, Italy	Apr-10	H. Breitkopf
O. passionis	Larzac, France	May-10	R. Romolini
O. promontorii	Mte. St. Angelo, Gargano, Italy	Apr-10	R. Souche
O. sipontensis	Siponto, Puglia, Italy	Mar-10	R. Romolini
O. sphegodes (CA)	Vesuvio, Campania, Italy	Mar-09	H. Breitkopf
O. sphegodes (GAR)	Capoiale, Gargano, Puglia, Italy	Mar-09	H. Breitkopf
O. splendida	Le Muy, France	Apr-10	R. Souche
O. tarquinia	Monte Argentario, Tuscany, Italy	Mar-08	G. Tosi
O. tyrrhena	Marina di Castagneto, Tuscany, Italy	Apr-09	H. Breitkopf

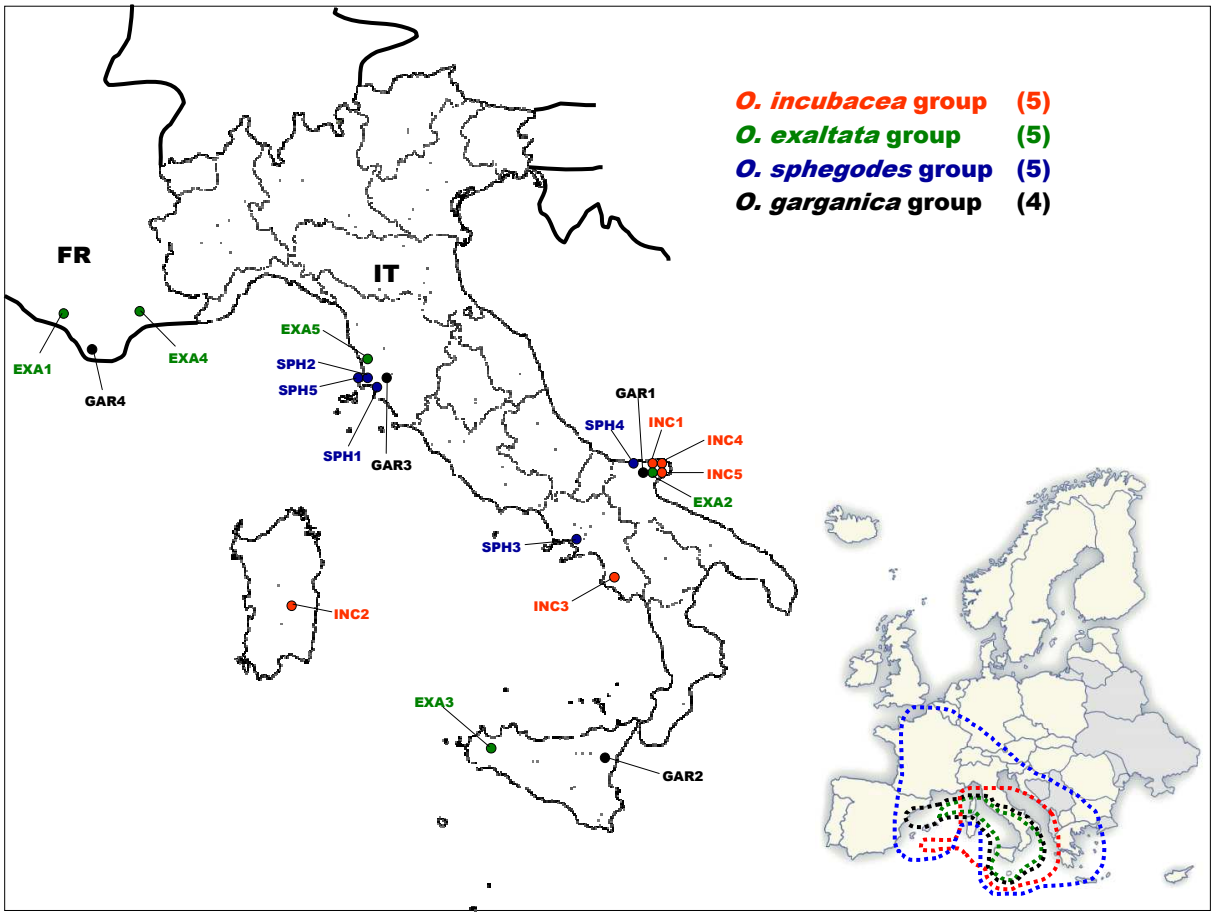

Fig.2 – Sampling locations in Italy and France (big map). Lower right map: species group distributions in Europe.

PCR & SEQUENCING

Newly designed and previously published primers were tested using polymerase chain reaction standard protocols and a 2720 Thermo Cycler (Applied Biosystems) with annealing temperatures ranging between 45°C and 63°C. With each primer couple at least three PCR's in a range of +- 5°C difference from the predicted melting temperatures were performed. Cycling conditions were as follows: 3 min initial denaturation at 94°C, followed by 35 cycles of 94°C 45 sec; TA 45 sec; 72°C 90 sec and a final extension of 72°C 7 min; 4°C hold. Annealing temperatures were 58°C for *ACS*, *FAD* and *MYB*, 59°C for *BGP*, 60°C for *LFY* and 65°C for *CAD*.

All PCR reactions were performed in a final volume of 25µl containing 50 – 100 ng DNA template, 10 pM of each, forward and reverse primer, 200µM of each dNTP, 2µl of 10x Taq buffer (50 mM KCl, 10 mM Tris-HCl pH 9), 0.5 µl 1.5mM MgCl2 and 0.5 U of Taq polymerase (Pharmacia, Amersham Biotech). PCR

products were separated on a 1,5 % agarose gel stained with ethidium bromide (0.3 mg/l) and photographed under UV light using a Gel Doc 2000 system (Biorad). Only primers that yielded discrete bands were further investigated; those that yielded a smear or no amplification were discarded. Products with multiple bands were separated in a 1.5 % TBE agarose gel, excised and purified using a kit. Those products that gave single bands were purified using IllustraTM GFX PCR DNA Purification Kit (GE Healthcare). Quality and concentration of the purificates was checked with a NanoDropTM 1000 Spectrophotometer (ThermoScientific). Amplificates were further amplified using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc.) and purified following the ethanol-sodium acetate precipitation protocol provided with the kit. Sequencing took place on 3130 and 3130xl Genetic Analyzers (Applied Biosystems, Inc.). Sequences were then analyzed with Sequence Analyzer 5.2 (Applied Biosystems, Inc.) and the chromatograms read out with Sequence Scanner 1.0 (Applied Biosystems, Inc.) or Chromas Pro (Technelysium Pty Ltd).

CLONING & PARALOGY ASSESSMENT

Heterozygosity and paralogy were expected to be issues when dealing with nuclear genes of the predominantly outcrossing genus Ophrys. If a sequence was partially unreadable due to existence of an indel in one of the two alleles, the PCR product was cloned into a bacterial vector (pGEM-T Easy, Promega) and inserted into competent *E. coli* cells by chemical transformation. Ambiguous SNP's were ignored as it was the aim to obtain consensus sequence. Cells were plated out on LB growth medium containing Ampicillin, allowing only the successfully transformed cells to grow colonies. Colonies were repicked to new plates for backup and storage. A small portion of the colonies was used as template for a PCR with the conditions following the manufacturer's protocol using the primer couple T7 / SP6 provided with the cloning kit (pGEM-T Easy, Promega). Sequencing was done as described above, but using the primers of the marker in question. Five to ten clones per accession were sequenced. If more than 2 alleles were detected in the cloning procedure the primers were identified to amplify at least one other copy of a gene/gene family and consecutively excluded. As *Ophrys* is generally diploid, no more than two alleles of any gene should be gathered from a single individual. The few species that have been

shown to be tetraploid are not included in this study. Cloning was applied for unphasing hetereozygotes in *ACS, BGP, CAD and FAD*.

We detected a notable haplotype differentiation in some genes, i.e. the obtained haplotypes could be classified into two or more groups regarding sequence similarity. To rule out the possibility that our PCR primers amplify paralogs, additional to the cloning procedure differential primers for the two most prominent sequence types/haplotype groups were designed for three genes (*CAD*, *FAD6, GPX*) for amplification and sequencing. PCR conditions were as described before with an annealing temperature of 59°C. We tested seven species with the new primers. Again, no more than two alleles per species could be detected.

Tab.3. – Primers used in this study and their respective annealing temperatures. *= nested haplotype group specific primers for paralogy assessment. **= from Cantone et al. (XXXX). All other primers can be used for both, PCR and sequencing

ACS-F	AGGTTGAGATTGCATTTGTGG	58°C
ACS-R	TTCAACAGCTTTTCTTTCATCG	
BGP-F	GCTGGCAACAATAGGATCTCCA	59°C
BGP-R	ACTGAGGAGCTTCCATCTAC	
CAD-F	CTACTTCTTCGGCGAGGCTAC	65°C
CAD-R	AGATGCTGTATGGAAGACACC	
CAD-1F*	TGCATTTTGAATTCTGTTACTTAT	59°C
CAD-1R*	AATCAGCTCTATACAAATGCA	
CAD-2F*	ACATTTTGGATTGTGTTACTT	59°C
CAD-2R*	AAATCAGCTCTAAACAAATGCAA	
FAD-F	ATATCACGCTCAGAGACATTATTACAAC	58°C
FAD-R	ATATGTCTTCCACCAACTTGTTCTTTG	
FAD-1F*	CTAGGCTTTGAACGTATCTCTTA	59°C
FAD-1R*	GACCTATGCGCACAATCATGA	
FAD-2F*	CTAGGCTTTGAACTTATCTCTTG	59°C
FAD-2R*	GACCTATGCGCACAATCATGG	
GGPS-F	AGAGGTTGAGGCGGTATGC	58°C
GGPS-r	CTTCAGCTGCAACTTGGCCT	
GPX-F	GTGCTGATTTTGGGTTGAGG	58°C
GPX-R	TTGATTTGAGGAACTTGTAGATGG	
GPX-1F*	CTTGGGAGTTGGGATAAGATT	59°C

GPX-1R*	TAATTGGCTGCAAGCATTCCC	
GPX-2F*	CTTGGGAGTTGGGATAAGATG	59°C
GPX-2R*	TAATTGGCTGCAAGCATTCCT	
MYB-F	GGAATTCCCTTGCTCTTTGTGC	58°C
MYB-R	GAGGTAATTGAGCCACCGAAGC	
OMT-F	ATGTCGAAGGAGATGTGTTTGC	58°C
OMT-R	CGCTCCAGTCATGAAGAATCC	
Orc*PI*-F	ATGGGGCGGGGAAATACGGAG	59°C
Orc*PI*-R	TCTCAGCATCTTCAAAAAATC	

SEQUENCE EDITING & ALIGNMENT

Sequences were assembled and aligned with the software BioEdit (Hall 2001) using the implemented ClustalW algorithm (Thompson et al. 1994), or manually with MacClade (Maddison & Maddison 1992). As computerized algorithms have been found to be inaccurate (Morrison 2009), the alignments have been checked by eye for apparent mistakes and manually corrected to minimize homoplasy. The alignment followed a two step procedure in which (1) all ingroup sequences were aligned to each other, followed by (2) alignment of the outgroup. Regions that could not be unambiguously aligned were discarded prior to phylogenetic analyses.

BASIC POPULATION GENETIC ANALYSES

The generation of polymorphic sites files (DNASP, Librado & Rozas 2009) allowed a first assessment of haplotype diversity by eye (see Fig.3 for an example). Levels of sequence polymorphism (n, L, S, Pi, ThetaW), haplotype diversity (n, h(div)), neutrality (Tajima's D) and linkage disequilibrium tests, amongst others, were inferred with DNASP, for all genes and the four predefined species groups, both as single and combined groups analyses. To assess if the observed parameter values deviated from predictions based on the proportion of segregating sites in the sequences (S) and an estimator of recombination (R) for each species group (R = gene length x Theta W), we calculated these values with 1000 coalescent simulations (DNAsp) under the infinite-sites model.

We calculated nucleotide diversity within (Tab.7) and between groups (Tab.8). Divergence was assessed by pairwise comparisons of silent substitution rates of the species groups (Tab.4).

```
•    INC1a  GTGGTGGCGTCTAGTCACTCGCGTGCAACTTAGCGG
•    INC1b  CGA..TATAC.AT.CAT.CT.T.A.TGCTG...T..
•    INC2a  .....................T..............
•    INC2b  .....................T..............
•    INC3a  ....................................
•    INC3b  CGA..TATAC.AT.CAT.CT.T.A.TGCTG...T..
•    INC4a  CGA..TATAC.AT.CAT.CT.T.A.TGCTG...T..
•    INC4b  CGA..TATAC.AT.CAT.CT.T.A.TGCTG...T..
•    INC5a  CGA..TATAC.AT.CAT.CT.T.A.TGCTG...T..
•    INC5b  ..............................T..

•    EXA1a  CGA..TATAC.AT..AT.CT.T.A.TGCTG...T..
•    EXA1b  CGA..TATAC.AT.CAT.CT.T.A.TGCTG...T..
•    EXA2a  CGA..TATAC.AT.CAT.CT.T.A.TGCTG...T..
•    EXA2b  .....................T....GC.....T..
•    EXA3a  .....................T..............
•    EXA3b  CGA..TATAC.AT.CAT.CT.T.A.TGCTG...T..
•    EXA4a  CGA..TATAC.AT.CAT.CT.T.A.TGCTG...T..
•    EXA4b  ..............................T..
•    EXA5a  ....CT..CC.A.A..TT..TG.A..G...A...AA
•    EXA5b  ...ACT...CAA....T...TTC......G.G..AA

•    SPH1a  CGA..TATAC.AT..AT.CT.T.A.TGCTG...T..
•    SPH1b  CGA..TATAC.AT.CAT.CT.T.A.TGCTG..AT..
•    SPH2a  .....................T..............
•    SPH2b  .....................T..............
•    SPH3a  CGA..TATAC.AT.CAT.CT.T.A.TGCTG...T..
•    SPH3b  CGA..TATAC.AT.CAT.CT.T.A.TGCTG...T..
•    SPH4a  CGA..TATAC.AT.CAT.CT.T.A.TGCTG...T..
•    SPH4b  CGA..TATAC.AT.CAT.CT.T.A.TGCTG...T..
•    SPH5a  CGA..TATAC.AT.CAT.CT.T.AATGCTG..AT..
•    SPH5b  CGA..TATAC.AT.CAT.CT.T.A.TGCTG...T..

•    GAR1a  CGA..TATAC.AT..AT.CT.T.A.TGCTG...T..
•    GAR1b  CGA..TATAC.AT.CAT.CT.T.A.TGCTG...T..
•    GAR2a  CGA..TATAC.AT.CAT.CT.T.A.TGCTG...T..
•    GAR2b  CGA..TATAC.AT.CAT.CT.T.A.TGCTG...T..
•    GAR3a  CGA..TATAC.AT.CAT.CT.T.A.TGCTG...T..
•    GAR3b  ....................................
•    GAR4a  CGA..TATAC.AT.CAT.CT.T.A.TGCTG...T..
•    GAR4b  CGA..TATAC.AT.CAT.CT.T.A.TGCTG...T..
```

Fig.3 – Polymorphic sites file (DNAsp) of the FAD gene dataset. 36 of 435 bp variable. Two largely different types of alleles with only little within variation; EXA5 alleles more derived.

COALESCENT PHYLOGENETICS & POPULATION STRUCTURE ANALYSES

We used the software BEST (Bayesian Estimation of Species Trees, Liu 2008) that integrates a coalescent theory based algorithm into the MrBayes program, to infer species trees from allelic sequence data. The single gene datasets were combined into a partitioned supermatrix. The combined dataset had a length of 5429 characters. The best fitting model of molecular evolution for each gene was chosen using the Akaike information criterion (AIC) as given in the output of MrModelTest 2.3 (Nylander 2004). Selected models were: *ACS*: F81+I+G, *BGP*: HKY+I, *CAD*: HKY+I, *FAD*: HKY+G, *GGPS*: GTR+I+G, *GPX*: HKY+I+G, *MYB*: GTR, Orc*PI*: HKY+G, *OMT*: HKY+I+G. *O. apifera* was used as the outgroup, as this species was shown to be genetically distinct and to be sister to the *O. sphegodes-holoserica* crown group. For the Bayesian analysis two consecutive runs of 10 million generations were performed on an external computer cluster (CBSU BioHPC, Cornell University, USA). The best species tree was compiled by the software (see: Liu 2008 for details).

Population structure was assessed using the programs Structure (Pritchard et al. 2000) and Structurama (Huelsenbeck et al. 2011). For the Structure analysis genotypes were assigned using the software DNASP (Librado & Rozas 2009), and coded into a numerical matrix. Prior to the analysis the most likely k (= number of populations) was estimated following the method of Evanno et al. (2005). Two analyses using the admixture model and different values of k (2 or 4) with 100k replicates and a burn-in of 10% were conducted. For the Structure bar-plots, individuals were aligned using their q-values.

We also tried to infer the most likely number of populations and species clustering in our sample using the software Structurama. Genotypes were coded into a numerical matrix as described before. The program was run for different values of k (k=1-20) and 10k generations to obtain posterior probabilities for each k. Furthermore, the program can be run for a fixed value of k to assign species to a predefined number of populations. We assumed four populations, equivalent to our four species groups. We used following settings: 100k generations, four MCMC chains, admixture, k=4.

HAPLOTYPE GROUPS DIVERGENCE TIME ESTIMATES

A strong haplotype differentiation was found in *CAD* and *FAD*, to a lesser extent in *BGP*, *GPX* and *OMT* (see Fig.3 for an example). We calculated divergence time estimates on the basis of published mutation rates for nuclear genes, generation times of *Ophrys* plants, and substitution rate estimates for our nuclear gene markers. The single genes' substitution rates were calculated as: (median number of intron differences between HT-groups [bp] / intron length [bp]) x introns' relative proportion [%]) + (median number of exon differences between HT-groups [bp] / exon length [bp]) x (1 – introns' relative proportion [%])*; (*= exon/intron's relative proportions derived from surveying the *A. thaliana* reference gene). As we investigate divergence of two separately evolving lineages, the genes' substitution rate was multiplied with only 50% of the mutation rates values published for nuclear genes (Wolfe et al. 1989, Ossowski et al. 2010), using their range extremes as upper and lower values. As the result is given as mutation rate per number of generations, we multiplied them with a generation time of *Ophrys* [in years] to obtain a more descriptive estimator. Though, little is known about the temporal dimensions between reproduction, seed dispersal, germination, vegetative growth, until flowering and newly reproduction. All together these processes constitute the steps of the reproductive cycle, known as generation time. The time from sowing to flowering under artificial conditions was stated to be little less than three years for several species of *Ophrys* (http://www.lidaforsgarden.com/Orchids/ophrys_eng.htm). We assumed little longer times under natural conditions, i.e. 3 – 5 years as factors for our estimates.

RESULTS

SEQUENCING

Neither in the cloning procedure, nor by application of differential primers more than two alleles were obtained for any accession. Unphased nuclear sequence data was generated for 9 genes, 19 ingroup accessions, and one outgroup (*O. apifera*). More than 1000 sequence reads were obtained to assemble about 220 sequences in total. An overview of heterozygosity levels over genes and accessions is given in Tab.5.

COALESCENT & POPULATION STRUCTURE ANALYSES

The BEST coalescent analysis was repeated three times. It resulted in three incongruent tree topologies, all of them with very low bootstrap support. A species clustering as expected from the morphology based taxonomic classification was not evident. In contrast, a moderate geographical clustering was evident in all analyses; i.e. species/accessions showed a trend for clustering when growing in sympatry, regardless of their taxonomic affinities (Fig.4). We found five geographic clusters, with four of them composed of two to three accessions from different species groups. It has to be noted that the observed clustering was not constant in our three analysis replicates. But the geographical clustering was in all cases obviously more apparent than a species clustering.
We estimated k (= numpops) for the combined genotype matrix following Evanno et al. (2005). The most likely number of populations was found to be two (k=2). Accordingly, Structure was run with k=2, and regarding our sampling scheme, with k=4.

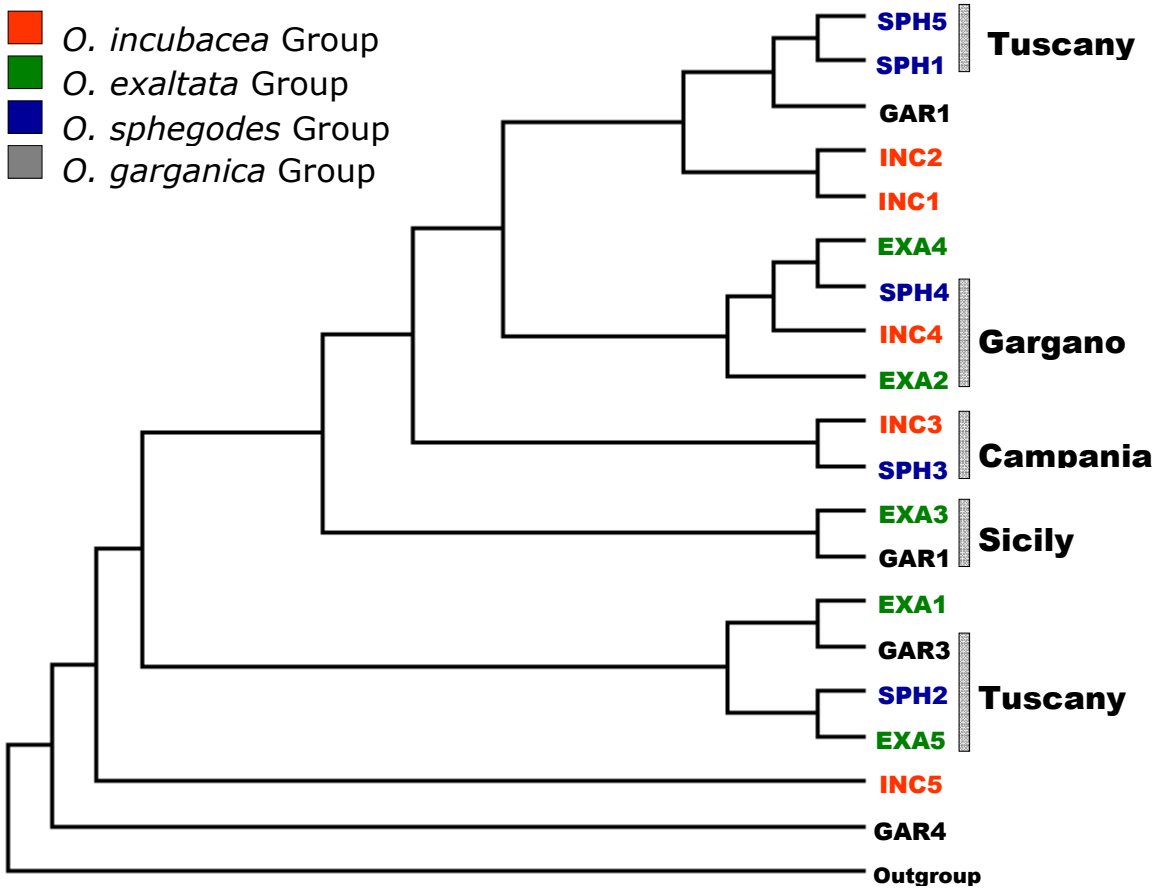

Fig.4 – Species tree. Coalescent theory based species tree obtained with BEST (Bayesian Estimation of Species Trees). $10x10^7$ generations runtime, bootstrap at all nodes always <=50%.

The results are output as bar-plots (Fig.5). For k=4 no significant clustering could be detected. But for k=2 a slight species clustering is apparent. All accessions of the *O. incubacea* group cluster together in one population, while the accessions of the *O. garganica* group cluster in the other population or as individuals with mixed ancestry. Individuals from the *O. exaltata* and *O. sphegodes* groups are scattered over the bar-plot

Structurama uses the same method as Structure to infer species clustering for a fixed k. As expected the program then inferred a similar confusing clustering as Structure for k=2 or k=4 (data not shown). In a second step we used to program to calculate posterior probabilities for different values of k. The k with the highest pp value is the most likely number of populations. But we obtained ever increasing values for k=1 up to k=20. Therefore no k value could be estimated using our data.

ALLELIC VARIATION & HAPLOTYPE DIVERGENCE ESTIMATION

In some genes we observed two largely divergent haplotype groups with only little apparent recombination between those allele types, and little to no variation within a single haplotype group. We use a descriptive population genetic approach to gather information about within and between species groups diversity for the employed genes, as well as to determine the observed haplotype groups with statistical methods.

Within group statistics: Nucleotide diversity (Pi) is lowest in *ACS* (0.002), and highest in *CAD* (0.048), followed by *FAD* (0.020), *GPX* (0.017) and *OMT* (0.017). The latter four genes are exactly those genes with the most obvious haplotype structure; i.e. the high Pi values express the existence of two very divergent alleles, rather than

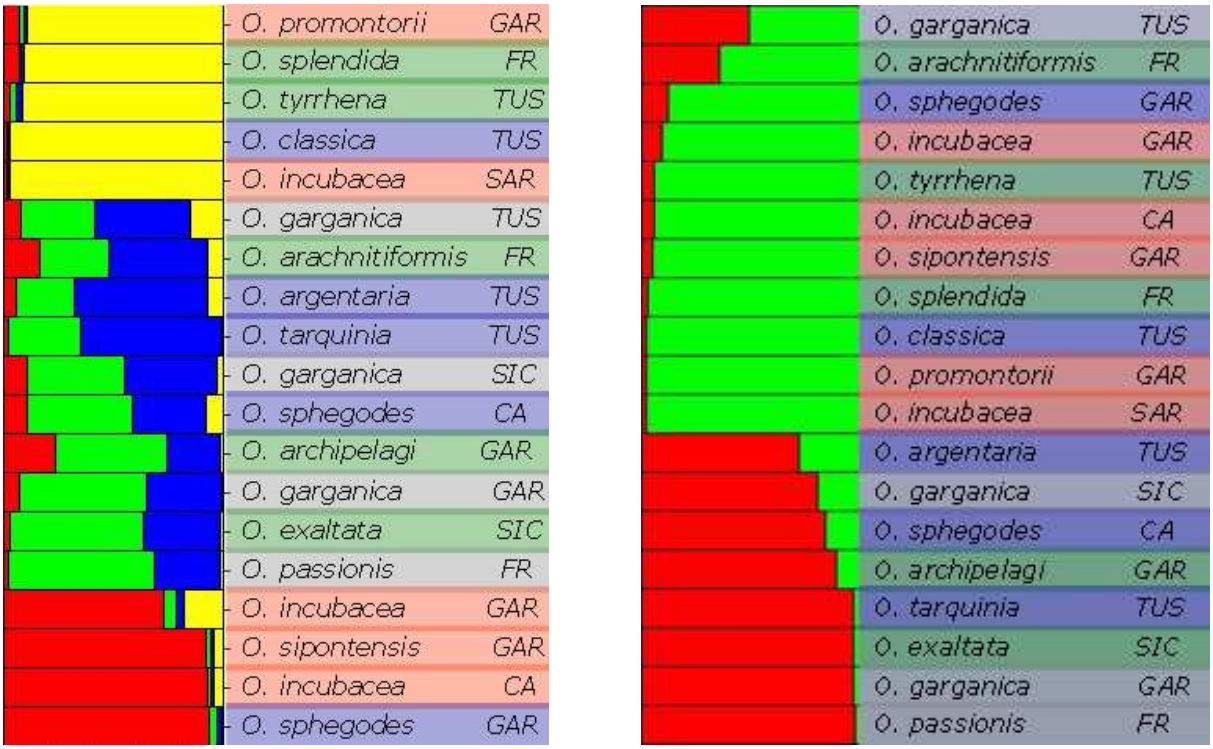

Fig.5 – Species clustering. Structure cluster analysis for k=4 (left) and k=2 (right). red – *O. incubacea* group, green – *O. exaltata* group, blue – *O. sphegodes* group, grey – *O. garganica* group. Sampling localities: GAR – Gargano, Puglia, It; TUS – Tuscany, It; CA – Campania, It; SIC

	INC-EXA	INC-SPH	INC-GAR	EXA-SPH	EXA-GAR	SPH-GAR	IN-OUT
ACS	0.001	0.002	0.001	0.002	0.002	0.003	0.017
BGP	0.017	0.018	0.017	0.014	0.014	0.016	0.035
CAD	0.076	0.044	0.098	0.064	0.055	0.069	0.079
FAD	0.053	0.047	0.046	0.050	0.048	0.026	0.084
GGPS	0.032	0.016	0.045	0.027	0.056	0.028	0.041
GPX	0.018	0.019	0.017	0.017	0.017	0.017	0.028
MYB	0.000	0.000	0.000	0.000	0.000	0.000	0.000
OMT	0.023	0.010	0.012	0.023	0.026	0.013	0.069
Orc*PI*	0.009	0.010	0.010	0.008	0.010	0.011	0.127
sum	0.228	0.165	0.247	0.204	0.227	0.184	0.480

Tab.4 – Divergence, pairwise group comparisons. Silent Substitutions (Ks JC-Silent / total sites).

phylogenetically informative polymorphisms. Therefore it doesn't surprise that in *CAD* and *FAD* the observed number of alleles (h) is much lower than the number of alleles to be expected (DNAsp coalescent simulations) based on the number of segregating sites. Similarly, the observed haplotype diversity (hDiv) in *CAD* and *FAD* is significantly lower than expected from the simulation. For the other genes the simulated values are similar to the observed ones, or deviate less strong from them; also the results of the Tajima's D test point out the deviation from neutral expectations in *CAD* and *FAD*, as in most species groups they are high, some of them significantly. Though it has to be said that Tajima's D and Zns values are here only used as rough estimators, because they are prone to error when calculated for small sample sizes. Summarized over all genes, nucleotide diversity (Pi) is highest in the *O.exaltata* group (0.147) and lowest in the *O. incubacea* group (0.078).

Species group statistics comparison: We averaged Pi over all sites and loci, made the six possible pairwise comparisons between our four species groups, and compared all the ingroup to the outgroup *O. apifera* (Tab.8). As expected, Pi was highest between the outgroup and all the ingroup (0.0132). Pi was only slightly lower in the comparisons EXA/SPH (0.0121) and SPH/GAR (0.0120), and lowest in INC/EXA (0.0083). Another way to assess divergence between the species groups is to make pairwise comparisons of only silent substitutions (Tab.4).

Averaged over all loci, following IN/OUT, INC/GAR and INC/EXA were the most divergent comparisons, INC/SPH and EXA/SPH the least divergent ones.

After paralogy had been excluded to be responsible for the unusual haplotype structure in the sample (see materials and methods), we proposed the recent coalescence of at least two lineages in the common ancestor of the investigated species group. The strong genetic differentiation among the haplotype groups indicates that they previously were separated over long time, while the bare differentiation within one haplotype group suggests recent coalescence. This raised the question, how long the two proposed ancestral lineages have been separated? *CAD* and *FAD* most prominently exhibited the occurrence of two strongly divergent alleles, to a lesser degree also *OMT*, *GPX* and *BGP*. We calculated estimates for all genes (Tab.6) as described in the materials and methods section. Estimates [in Ma] ranged from (13.1) 8.4 (5.2) for *CAD*, over (4.8) 3.1 (1.9) for *BGP*, to (2.0) 1.3 (0.8) for Orc*PI*.

Tab.5 – O. sphegodes group accessions used in this study and their zygosity level. Heterozygosity is marked as 'xx'. For multiple accessions of the same species the sampling region in Italy is given as abbreviation, i.e. GAR = Gargano, Puglia, SIC = Sicilia, TUS = Tuscany, SAR = Sardinia, CA = Campania.

#	*Species*	*BGP*Orc*PI*	*GPX*	*CAD*	*FAD*	*MYB*	*ACS*	*OMT*	Group
1	***O. arachnitiformis***	X	XX	XX	XX	XX	X	XX	XX	**EXA 1**
2	***O. archipelagi***	X	X	XX	X	XX	X	X	X	**EXA 2**
3	***O. argentaria***	X	XX	XX	X	XX	X	XX	X	**SPH 1**
4	***O. classica***	X	XX	X	X	X	X	X	XX	**SPH 2**
5	***O. exaltata s.str.***	XX	X	X	X	XX	X	X	X	**EXA 3**
6	***O. garganica GAR***	XX	X	XX	X	XX	X	X	XX	**GAR 1**
7	***O. garganica SIC***	XX	X	X	XX	X	X	XX	X	**GAR 2**
8	***O. garganica TUS***	XX	X	X	XX	XX	X	XX	XX	**GAR 3**
9	***O. incubacea GAR***	XX	X	X	X	XX	X	XX	XX	**INC 1**
10	***O. incubacea SAR***	X	XX	X	X	X	X	X	XX	**INC 2**
11	***O. incubacea CA***	X	XX	XX	X	XX	X	X	XX	**INC 3**
12	***O. passionis***	XX	X	X	X	X	X	X	X	**GAR 4**
13	***O. promontorii***	X	X	X	X	X	X	X	X	**INC 4**
14	***O. sipontensis***	XX	X	X	X	XX	X	X	XX	**INC 5**
15	***O. sphegodes CA***	XX	XX	X	X	X	X	X	X	**SPH 3**
16	***O. sphegodesGAR***	X	X	X	X	X	X	X	XX	**SPH 4**
17	***O. splendida***	XX	X	XX	X	XX	X	X	X	**EXA 4**
18	***O. tarquinia***	X	XX	X	XX	XX	X	XX	X	**SPH 5**
19	***O. tyrhena***	X	part	X	XX	XX	X	X	XX	**EXA 5**

DISCUSSION

Our aim was to shed some light on the issue of species delimitation, and to assess the degree of genetic separateness/togetherness in an ensemble of taxa that are differentially treated as separate species, subspecies, varieties or populations. Following the most conservative taxonomic treatment (Sundermann 1980) the 19 sampled accessions can be assigned to four subspecies of *O. sphegodes*, namely *ssp. arachnitiformis*, *ssp. atrata* (syn. *O. incubacea*), *ssp. garganica*, and *ssp. sphegodes*. For such an investigation on a hierarchical level

below that of good species, population genetic methods have to be invoked. We used two different approaches to infer species clusters. The coalescent theory based phylogenetic approach (BEST) yielded species clusters of spatial proximity, while the model-based approach (Structure) exhibited a slight clustering of individuals that are thought to be very closely related.

The BEST analysis inferred some clades that are entirely or for the most part composed of individuals from close-by or adjacent populations (Fig.4), while no clustering of putatively conspecific or very closely related taxa was found. This finding clearly indicates gene flow between *Ophrys* taxa in sympatry, even though they 'should be' reproductively isolated through different specific pollinators. The other possible explanation for this pattern is the repeated de-novo evolution of morpho-species, i.e. different lineages converge on the same phenotype due to adaptation to the same pollinator. Though, the authors favour the preceding hypothesis, as it is by far more parsimonious.

SPECIES GROUPS OR POPULATIONS

The *O. incubacea* (INC) and *O. garganica* (GAR) groups are the most divergent ones in our sample, as shown by Structure (Fig.5) and divergence analyses (Tab.4). The allele composition of these two groups is more homogenous than, and distant to the *O. exaltata* (EXA) and *O. sphegodes* (SPH) groups. On first sight, this finding contrasts with the fact that the latter two groups were shown to be the most strongly divergent groups in pairwise comparisons of the groups' nucleotide diversity (Tab.8). Furthermore, the *O. exaltata* group has the highest within-group nucleotide diversity (Tab.7). From the results of Structure and divergence analyses, INC and GAR were expected to show the strongest separateness in pairwise comparisons of Pi. This can be explained by looking at the allele distribution within our four groups and nuclear genes: SPH and EXA more often contain alleles of both, the two haplotype groups, as well as new alleles (see Fig.3 for an example). These two groups are therefore genetically more heterogeneous, and their alleles tend to cluster more often with themselves and the other two groups. Experiences from the field confirm this finding: INC and GAR are morphologically well differentiated and often more easily to distinguish from each other than EXA and SPH. But INC and GAR sometimes share morphological features of SPH. Again, this finding parallels the results from

the divergence analysis (Tab.4), where the couples INC-SPH and GAR-SPH were the two least genetically differentiated ones among all comparisons.
A clear differentiation in species or species groups based on the combined analysis of multiple nuclear genes that present the most variable markers under more than 100 tested ones, is therefore not possible. In fact, when only considering the observed allele sharing pattern and the lack of variation between the two allele types, one would expect our sample to come from only two recently hybridizing species.

DEMOGRAPHIC HISTORY

We proposed the coalescence of two genetically distinct lineages within a common ancestor of the sampled species groups to be responsible for the occurrence of two strongly divergent allele types. In turn, little genetic variation between these allele types points towards the recentness of the coalescence event. The high number of segregating sites in the two allele groups allowed a rough approximation of the time needed, to accumulate these differences, i.e. the divergence time. In this estimation we incorporated two factors afflicted by uncertainties: The generation time of *Ophrys* plants has not been investigated so far and we based them on experiences from artificial *Ophrys* cultures. Also the mutation rates of nuclear genes have large amplitudes of variability, as can be seen in our genes, where we find a continuum from no variation, over moderate variation, up to genes with strong haplotype structure. Nevertheless, the estimated divergence times for the four most haplotype structured genes range from 3.4 to 8.4 Ma, with a median value of 6.2 Ma. Even though these estimates are probably strongly biased by the aforementioned uncertainties, an effective separation of two *Ophrys* lineages for a few million years is highly plausible. Reasons for that can be either reproductive isolation or geographic isolation. We favour the latter explanation, as reproductive isolation barriers are expected to become stronger over time (e.g. Schluter 2001, Widmer et al. 2008). Reproductive isolation barriers in *Ophrys* are porous, so it can be assumed that they were even weaker in past times.
Our median divergence time estimate of 6.2 Ma closely falls together with the Messinian Salinity Crisis. The period of 5.8 to 5.3 Ma ago at the end of the Miocene was characterised by the repeated desiccation and flooding of the Mediterranean Basin caused by the closure and opening of the Street of Gibraltar.

Within the dry periods a desert like area covered the central part of the Mediterranean, which could have acted as an effective isolation barrier between separated lineages of *Ophrys*. The spatial isolation might have contributed to the evolution of strong reproductive isolation barriers that prevented the two lineages from gene flow for some millions of years. If this hypothesis holds true, it implies a recent breakdown of the isolation barrier, and an investigation of the allele composition in populations from the margin of today's distribution area could allow to detect the spatial origins of the two allele types based on their frequencies, and to identify possible refuge areas. The dating of major lineages of *Ophrys* would help to link cladogenesis and radiations to the geographic and climatic history of the Mediterranean region. The available methods to test for simple evolutionary models and infer time estimates/migration rates for periods of gene flow and/or isolation could not be applied to our data. This is due to properties of our nuclear gene data that violate some assumptions of the models/algorithms implemented in the respective software (IMa2, WH, Migrate), such as apparent recombination and the lack of fixed differences and gradual segregation of polymorphism among our four species groups. Applications to our data resulted in never converging runs or crude results.

SPECIES CONCEPTS FOR *OPHRYS*

The genus *Ophrys* constitutes a prime example for the differential approaches to species delimitation when largely different species concepts are taken into consideration. Let us review the different classifications: strict bio-species concept (Mayr 1942) → 1 spp.; phylogenetic species concept (Cracraft 1989) → 10 spp. (Devey 2008); inclusive phenetic/morphospecies (PM) concept (Cronquist 1978) → 16 spp. (Sundermann 1980) / 19 spp. (Faurholdt & Pedersen 2007); less inclusive PM concept → 49 spp. (Baumann & Künkele 1982); highly split PM/ethological species concept (Van Valen 1976), not consistently applied → 150 spp. (Devillers & Devillers-Terschuren 1994) / 252 spp. (Delforge 2006). Of those, the phylogenetic and the ethological species concept are based on objective criteria that are at least in theory consistently applicable: genetic divergence and isolation through specific pollinators, respectively. A diagnostic ethological concept would allow assigning morphological very similar populations with different specific pollinators the rank of species. Though, consistently applied it is mandatory, to identify the specific pollinator(s) over the whole

distribution range and flowering time, to exclude the existence of areas with pollinator sharing and hybridization with other sympatric *Ophrys* lineages, which is almost impossible.

Given the permeability of a reproductive system that is more or less based only on floral isolation, and the presumably strong fluctuation in the composition of the pollinating insect communities, gene-flow is likely to occur. And in fact, the existence of hybrids and hybrid zones is well documented (Souche 2008, Cortis et al 2008, Stoekl et al 2009). This parallels our finding of recent allele sharing between closely distributed species.

To our understanding, most entities of the *O. sphegodes/O.holoserica/O.scolopax* complex depict evolutionary significant units, or populations that temporarily build up reproductive isolation, but dynamically exchange genes with adjacent populations of closely related lineages. Our data suggests that these lineages are presumably incipiently speciating, and it cannot be predicted which of these lineages will coalesce in the future, and which will become good species. A big share of closely related *Ophrys* therefore presents transient species of a short-lived nature. We favour a more inclusive and conservative approach to species delimitation based on a phylogenetic species concept, as it employs a universally, and easily applicable objective criterion: separateness of lineages based on the degree of genetic divergence and the amount of gene-flow. The application of next-generation-sequencing data with hundreds of nuclear markers will allow for a more fine-scaled classification of *Ophrys* species, i.e. the detection of more than 10 minimum resolvable genetic units. Though, it will not help with the decision of how much gene-flow is tolerated between, or how much divergence is needed to make an *Ophrys* species.

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Tab.6 – Divergence time estimates. pink – intron/exon proportion in the *A. thaliana* reference gene (TAIR); violet – intron/exon ratio; grey – substitution rates in exon and intron; dark grey - overall gene substitution rate; turquoise – mutation rates per gene [in number of generations]; blue - mutation rates per gene [in million years]; lower right: medium estimates for the four most haplotype structured genes.

Gene	Gene length genomic TAIR [bp]	Gene length cds TAIR [bp]	Intron/exon proportion [percent]	Intron length [bp]	HT Intron differences [bp]	Intron SubstRate [per bp]	Exon length [bp]	Exon HT differences [bp]	Exon SubstRate [per bp]	Gene SubstRate [per bp]	# of Mill generations (SubstRate/MutRate) HIGH	MED	LOW	DivrgTimeEst (GenTime*GenNumber) [MillYrs] HIGH	MED	LOW
											0.0000000102			3		
											0.0000000128			4		
											0.0000000156			5		
CAD	1901	951	0.50	300	18	0.060	106	5	0.047	0.054	2.6	**2.1**	1.7	13.1	**8.4**	5.2
FAD6	2801	1301	0.54	230	20	0.087	222	2	0.009	0.051	2.5	**2.0**	1.6	12.4	**7.9**	4.9
OMT	2051	1101	0.46	695	48	0.069	11	0	0.000	0.032	1.6	**1.2**	1.0	7.8	**5.0**	3.1
GPX	1601	651	0.59	1004	37	0.037	71	0	0.000	0.022	1.1	**0.9**	0.7	5.4	**3.4**	2.1
BGP	5301	2501	0.53	844	26	0.031	132	1	0.008	0.020	1.0	**0.8**	0.6	4.9	**3.1**	1.9
ACS	4851	2001	0.59	619	4	0.006	49	1	0.020	0.012	0.6	**0.5**	0.4	3.0	**1.9**	1.2
GGPS	1451	1101	0.24	1	0	0.000	177	2	0.011	0.009	0.4	**0.3**	0.3	2.1	**1.3**	0.8
ORCPI	1106	371	0.66	735	8	0.0109	371	1	0.0027	0.008	0.4	**0.3**	0.3	2.0	**1.3**	0.8
MYB	2251	701	0.69	94	0	0.000	50	0	0.000	0.000	0.0	**0.0**	0.0	0.0	**0.0**	0.0
	23314	**10679**	**0.54**	**4522**	**161**	**0.036**	**1189**	**12**	**0.010**	**0.024**	**1.2**	**0.9**	**0.8**	**5.9**	**3.7**	**2.3**
											0.0000000051					
											0.0000000064		1 to 4	9.7	6.2	3.8

Tab.7 – Descriptive population genetics for seven genes with moderate to high heterozygosity levels in *Ophrys*. n=number of sequences, L=gene length [bp], S=segregating sites, Pi=nucleotide diversity, ThetaW=Watterson estimator, R=recombination level (L [bp] x ThetaW), P=probability of x(estimated) <= x(observed), avg=average value of x, h=haplotype number, hDiv=haplotype diversity, Rm=minimum number of recombination events, SS=synonymous substitutions, NSS=non-syn. subst., NCP=non coding polymorphisms. green filled columns: R, and coalescent simulated values of P and avg.

		Sequence Polymorphism					R	Neutrality Tests	R		Haplotypes	R		R			Rec	Linkage Disequilibrium	R		Syn. and Nonsyn. Substitutions			
	Spec. Group	n	L	S	Pi	Theta W	L* Theta	Tajima's D	P (D)	avg.	h	P (h)	avg.	h Div	P (hDi	avg.	Rm	ZnS	P (D)	avg.	SS	NSS	SS+NCP Pi(s)	SS+NCP #
ACS	INC	10	667	1	0.000	0.001	0.4	-1.112	0.420	-0.056	2	1.000	2.000	0.200	0.411	0.348	0	na	na	na	10.3	34.7	0.00032	631.3
	EXA	10	667	4	0.002	0.002	1.4	-0.822	0.198	-0.008	4	0.743	3.908	0.644	0.247	0.681	0	0.3345	0.628	0.323	10.3	34.7	0.00176	631.3
	SPH	10	666	6	0.002	0.003	1.8	-0.973	0.145	0.021	5	0.781	4.699	0.667	0.223	0.755	0	0.3345	0.634	0.324	10.3	34.7	0.03684	630.3
	GAR	8	667	4	0.002	0.002	1.5	-0.222	0.469	-0.086	5	0.997	3.763	0.857	0.956	0.710	0	0.0812	0.058	0.364	10.3	34.7	0.00232	631.3
	all	38	666	8	0.002	0.003	1.9	-1.398	0.055	0.015	10	0.981	7.862	0.647	0.164	0.740	2	0.0831	0.263	0.173	10.3	34.7	0.00179	630.3
BGP	INC	10	938	28	0.014	0.011	9.9	1.769	0.999	-0.056	6	0.103	8.055	0.844	0.020	0.941	2	0.4417	0.975	0.257	32.5	99.5	0.01643	837.5
	EXA	10	962	30	0.007	0.011	10.6	-1.662	0.006	-0.055	7	0.274	8.118	0.933	0.202	0.949	0	0.4335	0.963	0.256	32.5	99.5	0.00814	861.5
	SPH	10	957	31	0.014	0.011	11.0	1.040	0.962	-0.034	5	0.007	8.270	0.867	0.071	0.952	1	0.4591	0.966	0.254	32.5	99.5	0.01631	856.5
	GAR	8	959	36	0.015	0.014	13.9	0.058	0.544	-0.023	8	1.000	7.054	1.000	1.000	0.964	4	0.2144	0.189	0.278	32.5	99.5	0.01656	858.5
	all	38	934	52	0.014	0.013	12.4	0.098	0.576	-0.012	20	0.306	21.929	0.937	0.151	0.953	6	0.1444	0.982	0.115	32.5	99.5	0.01578	833.5
CAD	INC	10	258	0	0.000	0.000	0.0	na	na	na	1	na	na	0.000	na	na	na	na	na	na	na	na	na	na
	EXA	10	258	31	0.052	0.042	11.0	1.101	0.961	-0.048	6	0.082	8.193	0.844	0.023	0.947	0	0.5374	0.995	0.252	27.8	98.2	0.06987	158.8
	SPH	10	258	23	0.047	0.032	8.1	*2,333	1.000	-0.056	3	0.001	8.101	0.600	0.001	0.931	0	0.9407	1.000	0.265	27.8	98.2	0.06183	158.7
	GAR	8	260	24	0.035	0.036	9.3	-0.125	0.458	-0.035	4	0.017	6.667	0.643	0.002	0.941	0	0.6311	0.993	0.301	27.7	98.3	0.01941	160.7
	all	38	258	32	0.048	0.030	7.6	*2,167	0.998	-0.032	9	0.000	17.535	0.687	0.000	0.921	2	0.4794	1.000	0.134	27.7	98.3	0.0632	158.7
FAD6	INC	10	425	23	0.030	0.019	8.1	***2.644	1.000	-0.012	4	0.003	7.649	0.733	0.006	0.931	1	0.9215	1.000	0.267	52.6	169.4	0.05310	253.6
	EXA	10	425	34	0.035	0.028	12.0	1.153	0.918	-0.025	7	0.196	8.424	0.867	0.053	0.955	4	0.3898	0.956	0.249	52.6	169.4	0.06221	253.6
	SPH	10	425	24	0.020	0.020	8.5	0.003	0.491	-0.005	5	0.030	7.716	0.756	0.012	0.935	0	0.8143	1.000	0.264	52.6	169.4	0.03547	253.6
	GAR	8	425	23	0.014	0.021	8.9	*-1.739	0.001	-0.035	3	0.003	6.573	0.464	0.000	0.939	0	0.9503	1.000	0.309	52.6	169.4	0.02485	253.6
	all	38	425	36	0.020	0.020	8.6	1.048	0.882	-0.040	10	0.002	18.724	0.706	0.001	0.932	4	0.3899	1.000	0.127	52.6	169.4	0.04656	253.6
GPX	INC	10	908	33	0.016	0.013	11.7	1,295	0.985	-0.022	5	0.012	8.288	0,822	0.015	0.951	0	0,5989	0.997	0.250	4,3	16,7	0,01696	889,3
	EXA	10	908	38	0.017	0.015	13.4	0.830	0.931	-0.018	8	0.006	8.348	0,956	0.012	0.952	4	0,3443	0.920	0.243	4,3	16,7	0,01798	889,3
	SPH	10	932	31	0.014	0.011	11.0	1,500	0.993	-0.010	5	0.016	8.164	0,822	0.011	0.947	3	0,4992	0.986	0.252	4,3	16,7	0,01499	373,3
	GAR	8	937	31	0.016	0.012	12.0	1,684	0.999	-0.052	5	0.090	6.848	0,893	0.057	0.954	2	0,5186	0.979	0.288	4,3	16,7	0,01635	978,3
	all	38	889	48	0.017	0.013	11.4	1,143	0.960	-0.055	20	0.385	21.198	0,939	0.236	0.949	9	0,2211	0.989	0.120	4,3	16,7	0,01760	870,3
OMT	INC	10	653	17	0.009	0.009	6.0	0,044	0.554	-0.044	8	0.866	7.106	0,933	0.508	0.907	3	0,2042	0.248	0.280	0,7	8,3	0,00925	642,7
	EXA	10	608	47	0.030	0.027	16.6	0,185	0.612	-0.007	5	0.001	8.819	0,822	0.002	0.968	2	0,4104	0.987	0.227	0,7	8,3	0,03103	597,7
	SPH	10	657	21	0.009	0.011	7.4	-0.833	0.148	-0.006	6	0.200	7.521	0,844	0.048	0.927	5	0,2338	0.375	0.270	0,7	8,3	0,00954	646,7
	GAR	8	656	16	0.011	0.009	6.2	1,077	0.925	-0.008	6	0.248	7.388	0,929	0.070	0.921	0	0,4344	0.842	0.325	0,7	8,3	0,01169	645,7
	all	38	605	55	0.017	0.022	13.1	-0.948	0.072	-0.044	22	0.444	22.829	0,953	0.262	0.958	6	0,2356	0.994	0.115	0,7	8,3	0,01780	594,7
ORCPI	INC	10	962	22	0.008	0.008	7.8	-0.229	0.386	0.003	7	0.460	7.552	0,933	0.384	0.923	0	0,2476	0.459	0.274	86,0	283,0	0,01053	675,0
	EXA	10	962	16	0.004	0.006	5.7	*-1.737	0.005	-0.062	4	0.025	6.977	0,644	0.003	0.897	0	0,4514	0.932	0.281	86,0	283,0	0,00528	675,0
	SPH	10	958	24	0.006	0.009	8.5	-1.403	0.016	-0.054	9	0.925	7.763	0,978	0.938	0.934	1	0,1535	0.028	0.266	86,0	283,1	0,00866	671,9
	GAR	8	962	14	0.007	0.006	5.4	0.869	0.883	-0.014	5	0.365	5.887	0,893	0.301	0.895	0	0,3763	0.714	0.334	85,9	283,1	0,00945	674,9
	all	38	957	48	0.007	0.012	11.4	-1.631	0.002	-0.019	23	0.821	21.221	0,959	0.567	0.951	2	0,0715	0.999	0.121	86,0	283,1	0,00932	671,0

Tab.8 – Species groups comparison of nucleotide diversity (Pi). Species groups: I – *O. incubacea* group, E – *O. exaltata* group, S – *O. sphegodes* group, G – *O. garganica* group. avg=average Pi per site over all genes.

		ACS	*BGP*	*CAD*	*FAD*	*GGPS*	*GPX*	*MYB*	*OMT*	Orc*PI*	all	Avg.
I - E	all sites	667	936	258	425	177	907	144	605	962	5081	564.6
	Pi_all	0.200	13.553	0.000	12.665	0.712	14.802	0.000	6.068	7.398	55.398	6.155
	Pi_all (per site)	0.0003	0.0145	0.0000	0.0298	0.0040	0.0163	0.0000	0.0100	0.0077	0.0826	0.0083
I - S	all sites	666	936	258	425	177	896	144	653	957	5112	568.0
	Pi_all	0.200	13.553	0.000	12.665	0.712	14.802	0.000	6.066	7.398	55.396	6.155
	Pi_all (per site)	0.0003	0.0145	0.0000	0.0298	0.0040	0.0165	0.0000	0.0093	0.0077	0.0821	0.0108
I - G	all sites	667	938	258	425	177	901	144	653	962	5125	569.4
	Pi_all	0.200	13.554	0.000	12.665	0.712	14.803	0.000	6.066	7.398	55.398	6.155
	Pi_all (per site)	0.0003	0.0145	0.0000	0.0298	0.0040	0.0164	0.0000	0.0093	0.0077	0.0820	0.0108
E - S	all sites	666	955	258	425	177	896	144	608	957	5086	565.1
	Pi_all	1.625	13.752	12.134	8.487	0.712	12.956	0.000	5.709	5.981	61.356	6.817
	Pi_all (per site)	0.0024	0.0144	0.0470	0.0200	0.0040	0.0145	0.0000	0.0094	0.0063	0.1180	0.0121
E - G	all sites	667	957	258	425	177	902	144	608	962	5100	566.7
	Pi_all	1.467	14.039	9.035	5.929	0.428	15.181	0.000	7.466	6.320	59.866	6.652
	Pi_all (per site)	0.0022	0.0147	0.0350	0.0140	0.0024	0.0168	0.0000	0.0123	0.0066	0.1039	0.0117
S - G	all sites	666	957	258	425	177	985	144	656	957	5225	580.6
	Pi_all	1.625	13.752	12.134	8.487	0.428	14.381	0.000	6.114	5.981	62.903	6.989
	Pi_all (per site)	0.0024	0.0144	0.0470	0.0200	0.0024	0.0146	0.0000	0.0093	0.0063	0.1164	0.0120
I - O	all sites	666	892	224	425	177	821	143	604	950	4902	544.7
	Pi_all	1.126	12.961	9.831	11.122	1.308	11.642	0.000	10.262	6.356	64.607	7.179
	Pi_all (per site)	0.0017	0.0145	0.0439	0.0262	0.0074	0.0142	0.0000	0.0170	0.0067	0.1315	0.0132

"FLORAL ISOLATION IS THE MAIN REPRODUCTIVE BARRIER AMONG CLOSELY RELATED SEXUALLY DECEPTIVE ORCHIDS"

ABSTRACT

Floral isolation is an important component of pollinator-driven speciation. However, up to now, only a few studies have quantified its strength and relative contribution to total reproductive isolation. In this study we quantified floral isolation among three closely related, sympatric orchid species of the genus *Ophrys* by directly tracking pollen flow. *Ophrys* orchids mimic their pollinators' mating signals, and are pollinated by male insects during mating attempts. This pollination system, called sexual deception, is usually highly specific. However, whether pollinator specialization also conveys floral isolation is currently under debate. In this study, we found strong floral isolation: among 46 tracked pollen transfers in two flowering seasons, all occurred within species. Accounting for observation error rate, we estimated a floral isolation index ≥0.98 among each pair of species. Hand pollination experiments suggested that post-pollination barriers were effectively absent among our study species. Genetic analysis based on AFLP markers showed a clear species clustering and very few F1 hybrids in natural populations, providing independent evidence that strong floral isolation prevents significant interspecies gene flow. Our results provide the first direct evidence that floral isolation acts as the main reproductive barrier among closely related plant species with specialized pollination.

KEY WORDS: Floral odor, *Ophrys*, pollination, reproductive isolation, sexually deceptive orchids, speciation.

INTRODUCTION

Floral isolation is a form of pre-pollination reproductive isolation that can play an important role during the process of plant speciation (Grant 1994; Lowry et al. 2008; Kay and Sargent 2009; Schiestl and Schlüter 2009). Floral isolation can be mediated through the behaviour of pollinators (ethological isolation) or the morphology of the flower (mechanical isolation) and work in concert with other, later-acting reproductive barriers (Grant 1994; Fulton and Hodges 1999; Schemske and Bradshaw 1999; Ramsey et al. 2003; Aldridge and Campbell 2007; Schiestl and Schlüter 2009). The determination of the relative importance of different types of reproductive barriers among species has become a central topic in the study of speciation (Ramsey et al. 2003; Coyne and Orr 2004; Cozzolino and Scopece 2008; Lowry et al. 2008; Widmer et al. 2009). Previous studies have shown that in many plants, prezygotic isolation contributes more to total isolation than postzygotic isolation (Rieseberg and Willis 2007; Lowry et al. 2008; Widmer et al. 2009). In the absence of geographic barriers to gene flow (i.e., among sympatric species), floral isolation can be the most important prezygotic barrier. However, the relative strength of prezygotic and postzygotic isolation may differ between species, and may depend on the pollination system (Cozzolino et al. 2004; Cozzolino and Scopece 2008). In orchids, floral isolation has been suggested to be strong, because their associations with pollinators are often highly specific (Schiestl and Schlüter 2009). *Ophrys* L. is a genus of sexually deceptive orchids, which mainly occurs in the Mediterranean area. To attract pollinators, these orchids mimic the olfactory, visual, and tactile signals of the females of their associated pollinator insects, and thereby induce so-called pseudocopulations in males, leading to pollination (Kullenberg 1961; Kullenberg and Bergström 1976; Paulus and Gack 1990a,b; Schiestl et al. 2000). In this pollination system, floral odor is the key factor for specific pollinator attraction (Schiestl et al. 1999, 2003; Mant et al. 2005a,b; Peakall et al. 2010). One of the major characteristics of sexual deception is its high specificity, with each species of *Ophrys* only attracting one or very few species of male insects as pollinator(s) (Paulus and Gack 1990b). Therefore, different *Ophrys* species, which are mostly genetically compatible and crossable, are potentially isolated from each other due to ethological floral isolation, that is, the nonsharing of pollinator species (Ehrendorfer 1980; Paulus and Gack 1990b; Schiestl and Ayasse 2002; Scopece

et al. 2007; Schiestl and Schlüter 2009). Mechanical floral isolation is also present between some *Ophrys* species, mainly between the sections Pseudophrys and Ophrys (Kullenberg 1950; A° gren et al. 1984; Borg-Karlson 1990; Cortis et al. 2009). Among these groups, different *Ophrys* species can be pollinated by the same pollinator in sympatry, because pollinia are attached to different parts of the pollinator's body (head or abdomen), thus preventing pollen transfer between species. A recent study by Cortis et al. (2009), however, showed that crosspollination can occur in natural population despite mechanical isolation, which indicates that mechanical isolation in *Ophrys* may not be a very strong barrier to gene flow. Understanding the process of speciation and diversification in *Ophrys* orchids is challenging due to their high morphological variability, which can mean that it is often difficult to reliably identify species in the field. This is further complicated by the multiple and often highly divergent taxonomic treatments of the group. For example, the number of species in *Ophrys* listed by different authors ranges from 17 species (and 44 subspecies) (Sundermann 1980) or 19 species (Pedersen and Faurholdt 2007) to 250 species (Delforge 2006). Moreover, recent genetic and molecular phylogenetic studies of Ophrys showed low genetic divergence among species (Soliva et al. 2001; Soliva and Widmer 2003; Devey et al. 2008). The pattern of low genetic differentiation among species can be explained by two (nonexclusive) hypotheses: (1), the genus *Ophrys* may have undergone (a) recent radiation(s), or (2), there is frequent gene flow among species. Under the first scenario, *Ophrys* species-diversification is either due to pollinator shifts mediated by a change in key floral traits (such as floral odor bouquets; Mant et al. 2005b; Schlüter et al. 2009; Vereecken et al. 2010) or habitat adaptation. However, under scenario 1, if the time since species diversification is short, neutral genetic structure would not yet be expected to show a clear separation among species (Harrison 1991; Klein 1998). Under scenario 2, it is assumed that the strength of reproductive isolation among sympatric *Ophrys* species is weak, perhaps due to low fidelity of pollinators, therefore resulting in frequent gene flow among species, which reduces the genetic differentiation among species after their initial divergence (Soliva and Widmer 2003; Devey et al. 2008). One fundamental difference between these two scenarios is the assumed strength of floral isolation among sympatric *Ophrys* species: the first scenario assumes strong floral isolation, whereas the second scenario assumes relatively weak floral isolation. The

absolute pollinator specialization (i.e., the number of pollinators visiting each species) in *Ophrys* has previously been investigated (e.g., Paulus and Gack 1990b; Mant. et al 2005b).

However, the relative pollinator specialization (pollinator sharing, ethological floral isolation) and the resulting proportion of interspecific pollen transfer are still unknown. In this study, we directly tracked pollen flow within and among three sympatric and co-flowering, closely related *Ophrys* species, and quantified floral isolation as well as components of postmating reproductive barriers among these three species. Additionally, the genetic structure was investigated among species. Specifically, the following questions are addressed in this article: (1) How strong is floral isolation among sympatric *Ophrys* species? (2) What is the contribution of prepollination, postpollination prezygotic, and postzygotic isolation barriers to the total reproductive isolation among sympatric *Ophrys* species? (3) What is the proportion of hybrids in natural populations?

MATERIALS & METHODS

STUDY SPECIES

To most effectively address the question of the relative importance of the different putative isolation mechanisms in *Ophrys*, a set of species with the following criteria are needed: (1) Species should occur and co-flower in sympatry; (2) species should have the same ploidy level; (3) species should be closely related. According to these criteria, the species *Ophrys sphegodes* MILLER, *O. exaltata subsp. archipelagi* (GÖLZ & H.R.REINHARD) DEL PRETE, and *O. garganica* NELSON EX O. & E. DANESCH were chosen in this study, because these three species co-flower and co-occur sympatrically in Southern Italy, phylogenetic analysis indicates that these species are closely related (Devey et al. 2008), and ploidy levels of these species are expected to be the same and confirmed in this study (D'Emerico et al. 2005).

PLANT MATERIAL

The species *O. sphegodes*, *O. exaltata*, and *O. garganica* were identified based on floral morphology, according to criteria described by Mant et al. (2005b). At Capoiale (CAP), where all three species co-occur and co-flower, samples of these three species were collected in 2008 and 2009 for both scent and genetic analysis; at Marina di Lesina (MDL: 41◦54_N, 15◦20_E), where mostly *O. exaltata* and *O. garganica* co-occur and co-flower (and only very few individuals of *O. sphegodes* were found), these two species were collected in 2008 and 2009 only for floral scent analysis; at the more distant Foce Garigliano (FCG: 41◦13_N, 13◦46_E), where *O. exaltata* and *O. sphegodes* co-occur and co-flower (and *O. garganica* does not occur), these two species were collected in 2008 for both scent and genetic analysis. Each study area was about five hectares in size, and was estimated to contain 2000–3000 flowering plant individuals (counting all three species). For each sampled plant individual, a piece of leaf tissue was collected, and placed in a plastic bag filled with silica gel (Sigma, Buchs, Switzerland) for subsequent molecular analysis, and one labellum of an unpollinated flower was cut, placed in a 2 mL vial (Supelco, Sigma Buchs, Switzerland) and rinsed in 500 µL hexane (Fluka, Sigma Buchs, Switzerland) for 1min while gently shaking. Thereafter, the labellum was removed and all scent samples were stored at −28◦C until being analyzed by gas chromatography (GC). In total, 73 *O. sphegodes* (49 from CAP, 24 from FCG), 72 *O. exaltata* (48 from CAP, 24 from FCG), and 26 *O. garganica* (all from CAP) were sampled for genetic analysis; 100 *O. exaltata* (35 from CAP, 34 from MDL, 31 from FCG); 94 *O. sphegodes* (62 from CAP, 12 from MDL, 20 from FCG) and 56 *O. garganica* (30 from CAP, 26 from MDL) were sampled for floral odor analysis.

IN SITU MEASUREMENT OF FLORAL ISOLATION

We used an experimental approach with a plot design to measure floral isolation. The plots were set up in the field as follows, at the same localities as naturally occurring plants. Two individuals of each species were randomly positioned in each plot (six plants for each plot in CAP and MDL, four plants for each plot in FCG where O. garganica was absent from the natural populations). The distance between neighboring plants was 0.5 m. For each experiment, 20 plots were set

up along a transect through the habitat of the orchids. The distance between neighboring plots was 20 m, because the average pollinia-carrying distance of Colletes pollinators was estimated to be around 5 m (Peakall and Schiestl 2004). Plants for the plot experiments were picked from natural populations, each flower was checked for pollinia removal or pollen deposition, and pollinia were stained alternately with the dyes brilliant green, methylene aniline blue, orange G, and trypan red as described previously (Peakall 1989). The color used for each species was randomized between experiments to reduce potential effects of staining color on pollinator behaviour. The inflorescence was put in a water-filled 15-mL plastic tube placed in the ground. Pollinia removal and deposition of massulae were recorded three days after setting up the plots. Because *Ophrys* massulae are relatively small, and the assessment of their presence requires some experience in the field, there is a potential for observation errors to happen. Thus, to assess the observation error rate, in a subset of the experiments, plants were checked at several time points. For about half of the plant individuals used in the plot experiments, one unpollinated flower labellum was removed from the inflorescence to collect floral odor as described above. In 2008, two replicates of the experiment were performed at each of the following locations: CAP,MDL, and FCG (in total six experiments, 120 plots). In 2009, two replicates of the experiment were performed at locations CAP and MDL (in total, four experiments, 80 plots). The experiment at FCG was not repeated in 2009 due to the relatively poor overall pollinator activity at this location in 2008 (see Table 1). At each location where experiments were performed, the pollination success of naturally occurring plants in the surrounding area (within 20 m of the transect) was recorded at the end of the flowering season.

MEASUREMENT OF POSTMATING ISOLATION BARRIERS

Manual crosses were performed in spring 2010 in the greenhouse of the Department of Structural and Functional Biology, University of Naples Federico II. All crossed plants were collected from sympatric natural populations of the three investigated species at CAP, where we set up the plot experiments. To prevent uncontrolled pollinations, plants were placed in cages covered with a thin net prior to flowering. Pollination experiments were performed by removing pollinia by touching the viscidia with a plastic toothpick and placing them on the stigmas

of other plants of the same species (intraspecies pollinations), or of a different species (interspecies pollinations). Care was taken to pollinate no more than two flowers per individual to prevent the potential negative effects of over-pollination on fruit set and seed viability. All possible crossing combinations among *O. exaltata*, *O. garganica*, and *O. sphegodes* were performed bidirectionally (yielding a total of 78 crossings, see Table 2). Ripe fruits were collected and stored in silica gel. Seeds were then observed under an optical microscope with 100× magnification and assigned to two mutually exclusive categories: viable and inviable seeds, based on the presence or absence of embryos, respectively.

PLOIDY-LEVEL ANALYSIS

Differences in ploidy can provide an important barrier to gene flow. Although we expected all three study species to be diploid, with a chromosome number of 2n = 36 having been reported from *O. sphegodes* and *O. garganica* (Greilhuber and Ehrendorfer 1975; D'Emerico et al. 2005), the ploidy level of *O. exaltata* has not previously been reported. Furthermore, ploidy may also be variable within species and/or among different populations. Therefore, we investigated the ploidy levels of all three species in our study populations. Ploidy levels of the three species were analyzed using pollinia. Two pollinia of a single flower per individual were collected in spring 2010 from CAP and MDL populations. In total, 86 *O. sphegodes*, 71 *O. exaltata*, and six *O. garganica* samples were analyzed. Using flow cytometry, we analyzed the relative ploidy level for each individual separately. For sample preparation and analysis, we followed a two-step protocol (Doleˇzel et al. 2007). Two pollinia were chopped and mashed together with approximately 25-mm2 leaf material of *Phaseolus coccineus* (2n, 1C = 1.01 ± 0.4 pg; Bennett and Leitch 2005), which served as internal standard (IS), with a sharp razor blade in 1-mL ice-cold Baranyi's solution (0.1 M citric acid, 0.5% Triton X-100; Baranyi and Greilhuber 1995). After filtering the suspension through a 30 µm CellTrics® disposable filter (Partec GmbH, Münster, Germany), the filtrate was centrifuged (5 min,
380 × g, room temperature) using a Sorvall® RMC 14 centrifuge (Kendro Revco Lindberg Heraeus Sorvall, Asheville, NC). After removal of supernatant, nuclei were resuspended in 40 µL of ice-cold Baranyi's solution. One hundred and sixty micro liters of Otto II solution (0.4 M Na2HPO4) supplemented with DAPI (4_, 6-

Table 1. Summary of plot experiments. Plot experiments were set up in 2008 and 2009. CAP, MDL, and FCG refer to locations where experiments were set up. Number of total flowers refers to the total number of flowers from three species used in each plot experiment; Number of flowers with stained pollinia refers to number of flowers carrying stained pollinia—there were no flowers with unstained pollinia at the outset of the experiment, but some flowers without pollinia (either due to pollinators or handling by experimenter). Number of plants refers to total number of plants from three species in each plot experiment; pollinated flowers refers to number of flowers receiving pollinia; flowers pollinated by stained pollinia refers to number of flowers that received stained pollinia; pollinia removal refers to number of pollinia removed; interspecies pollinia transfer refers to number of interspecies pollinia movements observed in plots. Number of visited plants was counted as number of plants which lost or received pollinia.

Year	2008						2009				SUM
Plot	CAP1	CAP2	MDL1	MDL2	FCG1	FCG2	CAP3	CAP4	MDL3	MDL4	10
Number of total flowers	153	168	202	232	115	141	192	225	205	222	1855
Number of flowers with stained pollinia	146	154	165	205	96	119	182	208	195	218	1686
Number of plants	58	56	59	60	38	39	60	60	57	59	546
Pollinated flowers	14	7	25	5	13	3	10	27	20	7	131
Flowers pollinated by stained pollinia	5	2	8	4	3	0	0	10	11	3	46
Pollinia removal	38	16	76	13	14	10	16	46	48	22	299
Interspecies pollinia transfer	0	0	0	0	0	0	0	0	0	0	0
Visited plants	20	14	32	8	12	7	13	25	25	15	171
Stained pollinia on *O. sphegodes* flowers	2	0	1	0	2	0	0	6	3	1	15
Stained pollinia on *O. exaltata* flowers	0	1	5	4	1	0	0	1	7	2	21
Stained pollinia on *O. garganica* flowers	3	1	2	0	0	0	0	3	1	0	10

diamidino-2-phenylindole; final concentration: 4 μgmL−1) were added and relative fluorescence intensity was recorded using a Cell Lab QuantaTM SC-MPL flow cytometer (Beckman Coulter, Fullerton, Canada) with a mercury arc lamp. Only samples with pollinia peaks of at least 1000 counts and a coefficient of variation of less than 10% were analyzed. To determine relative ploidy level of the three species, the ratio between the median of pollinia peaks and the median of IS peaks was calculated.

Table 2. Fruit set formation ratio after hand pollination within and between species pairs. The ratio (%) was calculated by 100 x number of fruit sets/number of crosses performed. No significant differences were found between interspecies and intraspecies crosses.

Crossing type	Pollen donor (♂)	Pollen receiver (♀)	Number of crosses	Fruit sets	Ratio
Interspecies	*O. sphegodes*	*O. garganica*	10	10	100
	O. garganica	*O. sphegodes*	10	10	100
	O. exaltata	*O. sphegodes*	8	8	100
	O. sphegodes	*O. exaltata*	6	6	100
	O. exaltata	*O. garganica*	10	9	90
	O. garganica	*O. exaltata*	9	9	100
Intraspecies	*O. sphegodes*	*O. sphegodes*	8	6	75
	O. garganica	*O. garganica*	10	9	90
	O. exaltata	*O. exaltata*	7	7	100

FLORAL ODOR ANALYSIS

GC analysis was performed as described by Mant et al. (2005b) with 300 ng n-octadecane (C18) added to the floral extracts as an IS. One micro liter of each sample was injected into an Agilent 6890 GC at 50◦C, followed by opening of the split valve and heating to 300◦C at rate of 4◦C/min. An HP-5 column and flame ionization detector (FID) were used, and hydrogen was used as a carrier gas, with nitrogen as the makeup gas. For identification of compounds, several samples were re-analyzed by GC with a mass selective detector (GC/MSD; Agilent 5975) using the same oven and column parameters. Spectrum and retention time of compounds were compared with those of synthetic standards, that is, alkanes: nonadecane (C19), henicosane (C21), docosane (C22), tricosane (C23), tetracosane (C24), pentacosane (C25), hexacosane (C26), heptacosane (C27), octacosane (C28), nonacosane (C29); and alkenes: (Z)-7-heneicosene [(Z)-7-C21], (Z)-9-heneicosene [(Z)-9-C21], (Z)-7-tricosene [(Z)-7-C23], (Z)-9-tricosene [(Z)-9-C23], (Z)-7- pentacosene [(Z)-7-C25], (Z)-9-pentacosene [(Z)-9-C25], (Z)- 11-pentacosene [(Z)-11-C25], (Z)-12-pentacosene [(Z)-12-C25], (Z)-7-heptacosene [(Z)-7-C27], (Z)-9-heptacosene [(Z)-9-C27], (Z)-11-heptacosene [(Z)-11-C27], (Z)-12-heptacosene [(Z)-12-C27], (Z)-7-nonacosene [(Z)-7-C29], (Z)-9-nonacosene [(Z)-9- C29], (Z)-11-nonacosene [(Z)-11-C29], (Z)-12-nonacosene [(Z)- 12-C29], where (Z)-number indicates the cis double-bond position. For sources of standard compounds see Mant et al. (2005a). It is noted that the discrimination of (Z)-11- and (Z)-12-alkenes was not possible with the GC parameters used. The relative amount of each odor compound was calculated as the proportion of the total amount of all alkenes and alkanes of a chain length between 18 and 30 carbons.

GENETIC DIVERGENCE AMONG SPECIES

Genomic DNA was extracted using GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, Italy). The AFLP procedure was performed as described by Vos et al. (1995), with modifications as reported in Moccia et al. (2007) using fluorescent dye-labeled primers. An initial trial using 14 different primer combinations on four individuals each of *O. sphegodes* and *O. exaltata* was conducted to identify those primers that yield the highest number of easily

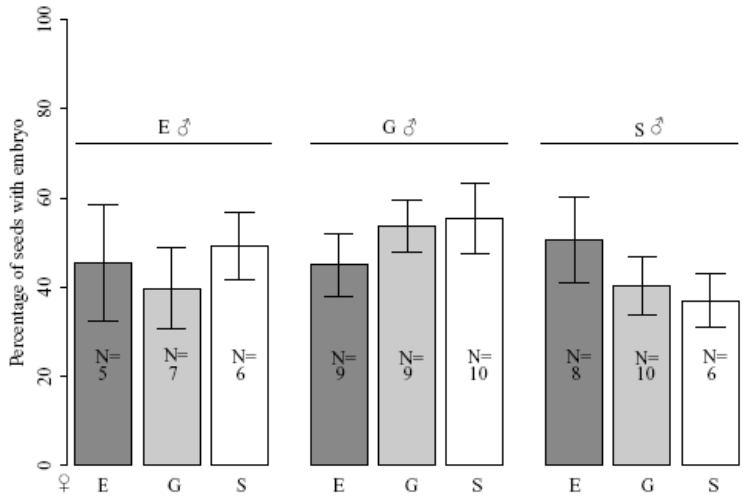

Figure 2. Presence of embryos for crosses among each species (E: *O. exaltata*; G: *O. garcanica*; S: *O. sphegodes*). No difference was found between interspecies and intraspecies crosses based on Student's *t*-test, ($P > 0.05$). Error bars depict the standard error of the mean.

detectable polymorphic peaks that were different between the two species. After the screening, six selective primer combinations were chosen: FAM-EcoRIAGC/ MseI-ACAC, NED-EcoRI-ACC/MseI-ACTG, HEXEcoRI- AGC/MseI-ATCG, FAM-EcoRI-ATG/MseI-CGG, NEDEcoRI- AAC/MseI-CGC and HEX-EcoRI-AGC/MseI-CCAA. For the restriction digestion, the enzymes EcoRI and MseI were used on a total of 250 ng of genomic DNA. Ligation of EcoRI and MseI adapters took place in the same reaction. Two microliter of the restriction-ligation product were used for a preselective PCR with primers having one selective base. For the successive selective PCR, 1 µL of a 1:10 dilution of the PCR product was used. Primers were the same as in the preselective PCR, but with three or four selective bases. Fragment separation and detection took place on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). GeneScan-500 LIZ (Applied Biosystems) was used as IS. Processing of the raw data and sizing of the fragments were done with Genemapper 3.7 software (Applied Biosystems). Absence or presence of AFLP bands was carefully scored by eye. To avoid artefacts, only AFLP markers that could be unambiguously scored over the whole dataset were included in the binary matrix. AFLP analysis was performed as two experiments at different dates and runs, and scored independently, preventing us from merging the two AFLP datasets. These two separate datasets were therefore analyzed separately: the first dataset contains 58 O. sphegodes (34 from CAP, 24 from FCG), 55 O. exaltata (31 from CAP, 24 from FCG), and 26 O. garganica (all from CAP) individuals; the second dataset contains 30 O. sphegodes (15 from CAP, 15 from FCG) and 32 O. exaltata (17 from CAP, 15 from FCG). In the second dataset,

Table 4. Pairwise population differentiation (Φ_{ST}) and overlap based on floral odor bouquet and AFLP among each species for two datasets. Dataset 1 contains all three species from CAP, whereas dataset 2 contains *O. exaltata* and *O. sphegodes* from CAP and FCG. The number of matches in floral odor was assigned based on the comparison between linear discrimination analysis of floral odor bouquet and species identification in the field. Matches in AFLP were assigned based on visual comparisons between principal coordinate analysis of AFLP markers and species identification in the field.

Species A	Species B	Φ_{ST}	Number of matches in floral odor/Total number of samples	Number of matches in AFLP/Total number of samples
Dataset 1				
O. sphegodes CAP	*O. garganica* CAP	0.044	0/173=0%	2/75=2.67%
O. sphegodes CAP	*O. exaltata* CAP	0.055	2/209=0.95%	1/97=1.03%
O. garganica CAP	*O. exaltata* CAP	0.064	1/138=0.72%	1/74=1.35%
Data set 2				
O. exaltata FCG	*O. sphegodes* CAP	0.074	5/68=7.35%	0/39=0%
O. exaltata FCG	*O. exaltata* CAP	0.063	121/126=96.03%	1/41=2.44%
O. exaltata in FCG	*O. sphegodes* FCG	0.068	5/68=7.35%	3/49=6.12%
O. sphegodes CAP	*O. exaltata* CAP	0.059	2/209=0.95%	0/32=0%
O. sphegodes FCG	*O. exaltata* CAP	0.073	4/116=3.44%	0/42=0%
O. sphegodes FCG	*O. sphegodes* CAP	0.059	150/151=99.34%	5/40=12.5%

both species from population FCG were the same individuals as in the first dataset.

EVALUATION OF THE STRENGTH OF INDIVIDUAL ISOLATING BARRIERS

The strength of each type of reproductive isolation barrier was calculated based on the quantitative approach suggested by Lowry et al. (2008) and Martin and Willis (2007). The floral isolation index was calculated based on the following formula: RIfloral =1− (observed/expected inter-species pollen flow)/(observed/expected intra-species pollen flow). The precision of this estimate of RIfloral is limited by the number of observed events in our experiments and any potential observation error introduced when checking plants for pollination or pollinia removal. Therefore, RIfloral was recalculated so as to account for these errors. Three of our experiments were checked twice, any error recorded, and these data were used to estimate the observation error rate. For example, on the same flower, massulae might have been recorded at the first but not on the second inspection, indicating that one of these two data points was probably erroneous. This error rate followed a pattern, that is the best fitted curve decreased exponentially with the number of experiments we performed, concordant with our expectation that observation error rate decreases as the observers' experience increases [ln(Error rate)=−0.25 × i ± SD, where i refers to the number of experiments performed by that time]. Based on this formula, the error rate for each experiment was estimated, and the floral isolation index recalculated by allowing observation error to occur at the

estimated error rate ± SD. We repeated this estimation procedure 1000 times to obtain a simulated distribution of floral isolation index values, and used this distribution to obtain mean and 95% confidence values for RIfloral. Because in orchids, female gametophyte development and fruit set formation usually happens after successful pollination with compatible pollen (Zhang and O'Neill 1993), the postmating prezygotic isolation index can be estimated as the proportion of fruit set (i.e., capsules) formed following interspecies pollinations, relative to the proportion of fruit set formed following intraspecies pollinations with each parental species (Scopece et al. 2007): RIpostmating−prezygotic =1 − (ratio of fruit set formed in interspecies crosses)/(average ratio of fruit set formed in parental intraspecies crosses). Similarly, the postzygotic isolation index was estimated by viable seeds and quantified based on the following formula (Scopece et al. 2007): RIpostzygotic = 1 − (proportion of viable seeds in interspecies crosses)/(average proportion of viable seeds in parental intraspecies crosses).

DATA ANALYSIS

Linear discrimination analysis (LDA) was used for analysis of floral scent based on relative amounts of hydrocarbons. Comparison of fruit set formation ratios among interspecies crosses and intraspecies crosses was performed by Fisher's exact tests. The significance of different seed viability among interspecies and intraspecies crosses was assessed using Student's t-test, after normality testing of the data distribution by the Shapiro test (Royston 1982). Statistical analysis of AFLP data was performed in FAMD 1.25 (Φ_ST and PCoA) (Schlüter and Harris 2006) and Hindex 1.41 (hybrid index; Buerkle 2005). Principal coordinate analysis (PCoA) was based on Jacquard's similarity coefficient. Correlations between pairwise floral odor and genetic distance was assessed in a Mantel test (10,000 permutations). Here, floral odor distance was calculated as a Euclidean distance, and genetic distance was calculated as 1 − Jacquard's similarity. Except for the analysis of AFLP data, all statistical analyses in this study were carried out in R 2.11.0 (R Development Core Team 2010).

RESULTS

POLLINATION SUCCESS

Among the natural populations, the average pollination success (defined as the percentage of pollinated flowers) was 10.8% in *O. sphegodes*, 18.8% in *O. exaltata*, and 3.2% in *O. garganica*, based on two flowering seasons (2008 and 2009). Furthermore, within each species, pollination success varied among populations (Fig. 1). No significant differences were found between the two years of observations. The overall patterns of pollination success in our plot experiments were similar to the natural populations. Pollination success in plot experiments was about 7.8%, 9.2%, and 4.1% for *O. sphegodes*, *O. exaltata*, and *O. garganica*, respectively. The population variation in pollination success was similar for natural populations and plot experiments, except for *O. sphegodes* in MDL where pollination success was much lower than in CAP (3.9%), whereas in plot experiments, the pollination success was similar to CAP (8.9%).

FLORAL ISOLATION

The flowering time of *O. sphegodes* and *O. exaltata* was similar, whereas the peak of *O. garganica* blooming was 1–2 weeks later (Xu, field observations). However, there was a broad overlap in flowering time, with around 70% of *O. garganica* flowers, and around 95% of *O. sphegodes* and *O. exaltata* flowers open during the experimental period. Of all 1855 flowers and 1686 stained pollinaria used in the experiments, 131 flowers (7.1%) were pollinated. Among these pollinated flowers, 46 flowers were pollinated with stained pollinia. Fifteen *O. sphegodes* flowers, 21 *O. exaltata* flowers, and 10 *O. garganica* flowers received stained pollinia (Table 1). All of the 46 pollination events with stained pollinia were within species, and we did not observe a single interspecies transfer. Thus, because no interspecies pollen flow was observed, the floral isolation index equals 1. Likewise, the simulated data incorporating observation error rates (see Methods) showed strong prepollination reproductive isolation among each species, RIfloral ≥ 0.98 (Table 3 and Fig. S1).

POSTMATING ISOLATION

Postmating, prezygotic isolation was estimated as the fruit set ratio after hand pollination. Most of the inter- and intraspecies crosses led to the development of capsules (Table 2). The lowest fruit set ratio was found for intraspecies crosses in *O. sphegodes*, although this was not statistically significant when compared to other crosses. Thus, the postmating prezygotic isolation index among each species was estimated to be very low. For species pairs *O. sphegodes/O. exaltata* and *O. sphegodes/O. garganica*, the isolation indices were negative (however, not significantly different from zero), which might indicate that interspecies crosses performed better than intraspecies crosses, and for *O. garganica/ O. exaltata*, the isolation indices was close to zero (Table 3). The proportion of seeds with embryos (viable seeds) was used to estimate the postzygotic isolation index. The number of seeds analyzed for each capsule was 324 ± 103 (mean ± SD). Among all fruits, the average percentage of viable seeds was 46.8 ± 21.8% (mean ± SD). We did not find a significant difference between any inter- or intraspecies crosses (Fig. 2). Similar to postmating prezygotic isolation, the mean postzygotic isolation index was also negative between species pairs *O. sphegodes/O. exaltata* and *O. sphegodes/O. garganica*, whereas for *O. garganica/O. exaltata*, the mean value index was slightly higher (Table 3). However, statistical analysis showed that none of these values were significantly different from zero.

PLOIDY-LEVEL ANALYSIS

No difference in the ploidy level was detected among the three species. The ratios of the relative inflorescence intensity between the pollinia and the IS are shown in Figure S2. All samples of these species showed similar relative genome size. It is most likely that all three species in our study are diploid, because previous studies showed that *O. sphegodes* and *O. garganica* are diploid (Greilhuber and Ehrendorfer 1975; D'Emerico et al. 2005).

FLORAL ODOR BOUQUET

The differences in floral odor bouquets among studied species were similar to those reported previously (Mant et al. 2005b). The major floral odor difference among the species was the proportion of different alkenes. By LDA, 236 of 250 samples (94.4%) were classified as the same species as they were identified in the field based on floral morphology according to the criteria described by Mant et al (2005b). The morphological/chemical identification mismatch rate between *O. sphegodes* and *O. exaltata* was 4.1%, between O. sphegodes and *O. garganica* 3.3% and between *O. garganica* and *O. exaltata* 0.64%.

GENETIC DIVERGENCE AMONG SPECIES

AFLP datasets one and two contained 242 and 322 markers, respectively. Genetic divergence among population pairs, as estimated by pairwise Φ _ST, was relatively low. For the first dataset, where all three species from CAP were analyzed, the lowest Φ _ST value (0.044) was found between *O. sphegodes* and *O. garganica*, and the highest (0.064) was found between *O. garganica* and *O. exaltata* (see Table 4). However, the differences among species pairs were very small. For the second dataset, where *O. sphegodes* and *O. exaltata* from both CAP and FCG were analyzed, the highest Φ_ST (0.074) was found between *O. sphegodes* in CAP and *O. exaltata* in FCG, whereas Φ _ST values between *O. sphegodes* in CAP and *O. sphegodes* in FCG, and between *O. sphegodes* in CAP and *O. exaltata* in CAP were lowest (0.059) (Table 4). Overall, Φ _ST values were low, within-species Φ _ST values being slightly lower than between-species values. Genetic structure among species was investigated by PCoA (Fig. 3). These analyses suggest that the genetic similarity between *O. sphegodes* and *O. garganica* is higher than between the species pairs *O. sphegodes/O. exaltata* or *O. garganica/ O. exaltata*. Although a few outliers were found for both CAP and FCG populations, the three species formed genetically separable clusters (Fig. 3A,B,C and Table 4). For the *O. sphegodes/O. exaltata* species pair, floral odor showed significant correlation with genetic distance for population FCG ($r = 0.42$, $P = 0.0001$), but not for population CAP ($r = -0.11$, $P = 0.69$). For species pairs *O. sphegodes/O. garganica* and *O. garganica/O. exaltata* in CAP, significant correlations were found in both ($r = 0.28$, $P = 0.0026$ and $r = 0.17$, $P = 0.036$,

respectively). For population CAP, no obvious F1 hybrids were found among the three species, as defined by a mean maximum likelihood hybrid index estimate between 0.4 and 0.6. In contrast, for population FCG, two samples were classified as potential F1 hybrids between *O. sphegodes* and *O. exaltata* according to the same criteria (Fig. 4). Both samples were classified as *O. sphegodes* based on floral odor discrimination analysis (Fig 3F). Overall, for samples from both populations (146 individuals), the percentage of F1 hybrids was very low (1.37%).

DISKUSSION

Reproductive isolation has been a central topic in the study of speciation (Coyne and Orr 1998; Moyle et al. 2004; Rieseberg and Willis 2007; Scopece et al. 2007, 2008; Schiestl and Schlüter 2009; Widmer et al. 2009). Here, we quantified three different kinds of reproductive barriers (floral isolation, postpollination prezygotic isolation, and postzygotic isolation), as well as ploidy level among three sympatric sexually deceptive *Ophrys* orchids using experimental approaches. Among these potential barriers, floral isolation was found to be very strong (RIfloral ≥ 0.98), whereas later-acting barriers were effectively absent in our study species. Furthermore, population genetic analysis showed a clear separation between species despite low genetic divergence, with few hybrids within natural populations. Our results shed light on the role of plant–pollinator interactions in the evolution of reproductive isolation and plant speciation. We suggest that pollinator adaptation, which conveys strong floral isolation, is the main driver of speciation in this plant group with highly specialized pollination.

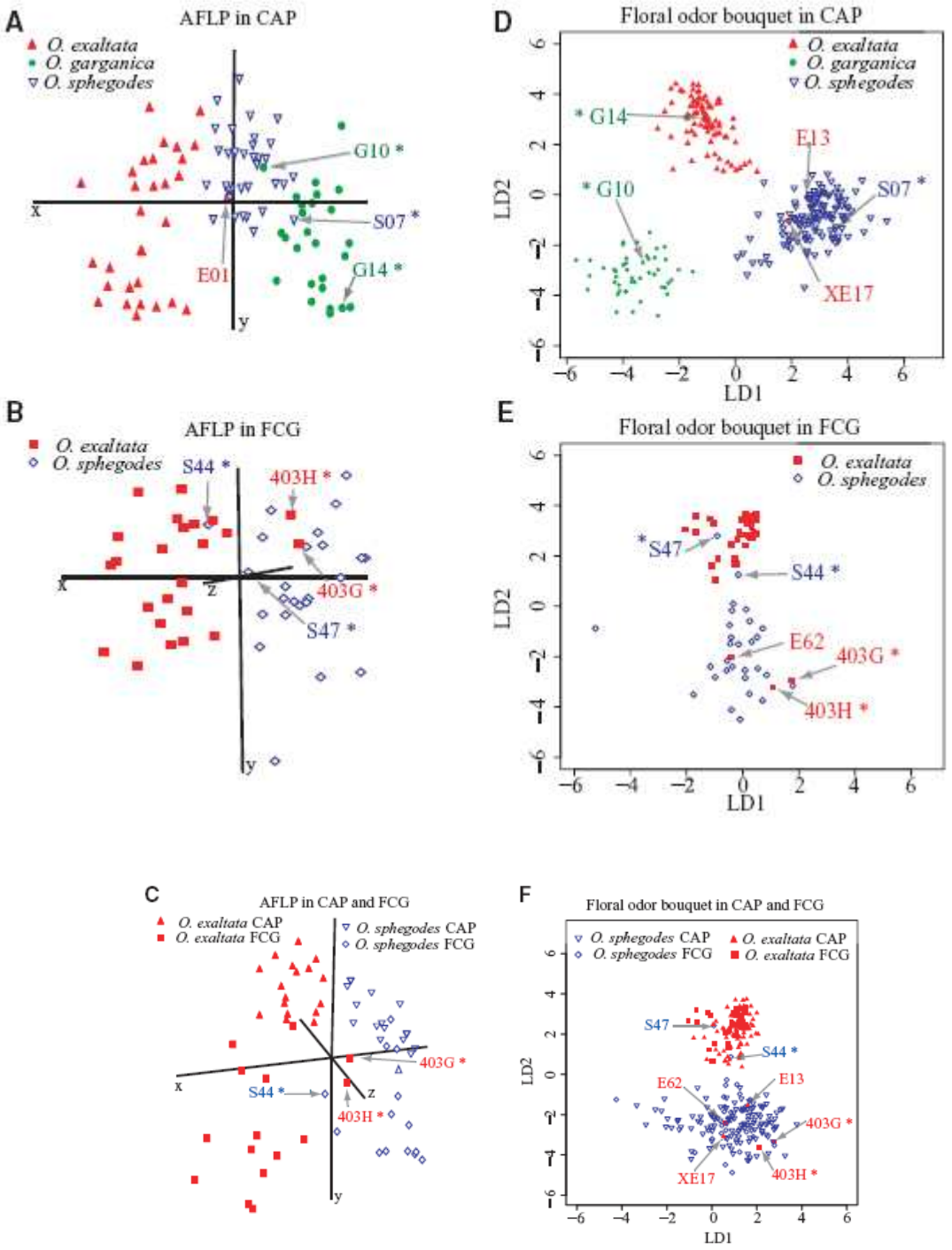

Figure 3. Plots of genetic structure (PCoA) and floral odor discrimination analysis for three species. (A) genetic structure of *O. exaltata*, *O. garganica*, and *O. sphegodes* in population CAP. Axis *x* and *y* represent 10.5% and 5.4% of variance, respectively; (B) genetic structure of *O. exaltata* and *O. sphegodes* in population FCG. Axis *x*, *y*, and *z* represent 12.1%, 8.1%, and 7.1% of variance, respectively; (C) genetic structure of *O. exaltata* and *O. sphegodes* in populations CAP and FCG. Axis *x*, *y*, and *z* represent 9.1%, 8.6%, and 5.9% variance, respectively; (D) floral odor bouquet of *O. exaltata*, *O. garganica*, and *O. sphegodes* in CAP, LD1, and LD2 representing 58.5% and 41.2% of trace, respectively; (E) floral odor bouquet of *O. exaltata* and *O. sphegodes* in FCG, LD1 and LD2 representing 55.9% and 44.1% of trace, respectively; (F) floral odor bouquet *O. exaltata* and *O. sphegodes* in FCG, CAP and MDL, LD1 and LD2 representing 55.9% and 44.1% of trace, respectively. An asterisk (*) indicates outliers for which both AFLP and floral odor bouquet were analyzed. Different colors indicate species (red: *O. exaltata*; green: *O. garganica*; blue: *O. sphegodes*).

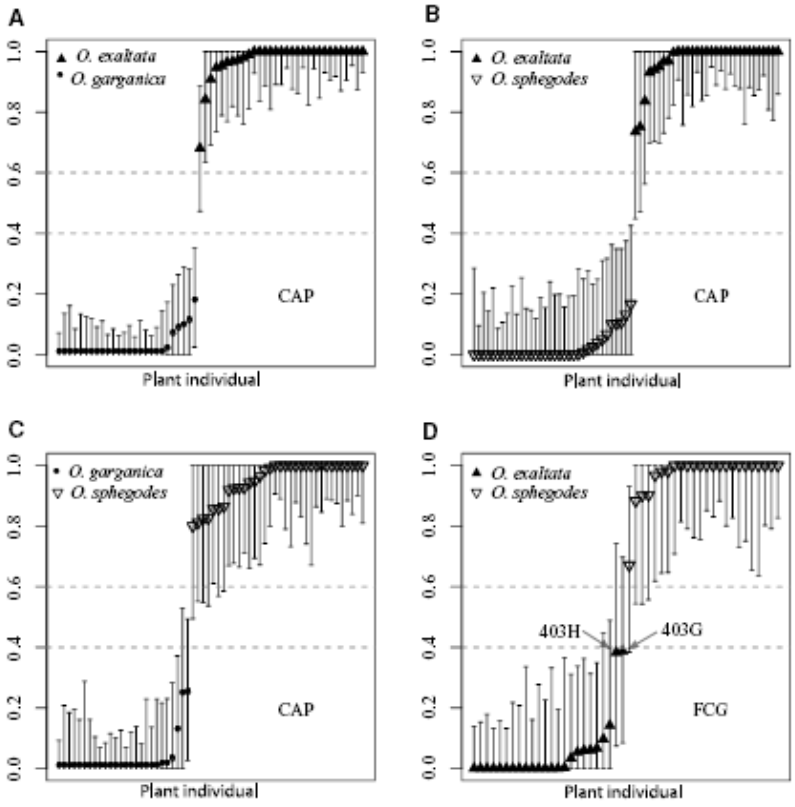

Figure 4. Hybrid index (with 95% confidence intervals) for individuals of each sympatric species pair. The two dashed lines indicate hybrid index $h = 0.4$ and $h = 0.6$ cutoff values for assigning putative F_1 hybrids. Sample names of two putative hybrids are indicated. (A) Individuals of *O. exaltata* and *O. garganica* in population CAP; (B) Individuals of *O. exalatata* and *O. sphegodes* in population CAP; (C) Individuals of *O. exaltata* and *O. sphegodes* in population CAP; (D) Individuals of *O. exaltata* and *O. sphegodes* in population FCG.

FLORAL ISOLATION IN PLANT SPECIATION

Floral isolation has been found in many plant–pollination systems, such as *Ipomopsis* (Grant 1992), *Mimulus* (Schemske and Bradshaw 1999; Ramsey et al. 2003), *Nicotiana* (Ippolito et al. 2004), Petunia (Hoballah et al. 2007), and *Silene* (Goulson and Jerrim 1997; Wälti et al. 2008), and meta-analyses indicate that floral isolation acts as a strong reproductive barrier in various families of flowering plants (Grant 1994; Lowry et al. 2008; Schiestl and Schlüter 2009; Kay and Sargent 2009; Schiestl, in press). However, there are few examples where floral isolation alone is sufficient to maintain species differentiation in sympatry (Kay and Sargent 2009; Schiestl, in press). In most studied cases, floral isolation acts together with other isolation barriers (postpollination isolation, ecogeographic isolation etc.; Lowry et al. 2008). Co-occurrence of floral isolation with other isolation barriers may be due to two reasons: (1) in most plant systems, it is unlikely that floral isolation has initially evolved in sympatry, because the shift to completely new pollinators may require changes in many

floral traits (but see Bradshaw and Schemske 2003 and Hoballah et al. 2007). Therefore, geographical or habitat-associated barriers would often be involved in the evolution of floral isolation; (2) once floral isolation is established, secondary isolation barriers can build up over time (Via and West, 2008; Matute et al., 2010; Moyle and Nakazato, 2010). However, to better understand the contribution of floral isolation to plant speciation, as well as its evolutionary patterns, cases in which only floral isolation is involved in the speciation process are particularly valuable.

REPRODUCTIVE ISOLATION AMONG SYMPATRIC *OPHRYS* SPECIES

As shown in our study, *Ophrys* may represent a case in which floral isolation is the most important barrier to gene flow among species with a large geographic overlap, sympatric occurrence, and flowering time overlap in their given habitats. Here investigated *Ophrys* species showed strong ethological floral isolation and a lack of postpollination isolation barriers. Our conservative estimation of the strength of floral isolation, which took into account the number of trackable pollination events and the observation error, showed that the floral isolation index among each *Ophrys* species pair was higher than 0.98. This estimation is consistent with our AFLP data, which indicate 1.37% (two of 146 samples) putative F1 hybrids between *O. sphegodes* and *O. exaltata*. Our finding of strong floral isolation is also consistent with expectations from pollinator-behaviour studies often indicating little pollinator sharing among co-flowering species pairs (Kullenberg 1961; Paulus and Gack 1990b; Mant et al 2005a, 2005b; Schlüter et al. 2009). Although we have only partially quantified postzygotic isolation barriers, it is clear from our results that the early acting floral isolation is the major (if not the only) reproductive barrier among closely related *Ophrys* species. Recently, floral isolation in *Ophrys* has come under scrutiny, because studies based on genetic markers argued that floral isolation in *Ophrys* might be weak and allow for considerable gene flow across species boundaries (Soliva and Widmer 2003; Devey et al. 2008). However, the data presented in these studies only allow for an indirect inference on floral isolation. The genetic pattern observed by Soliva and Widmer (2003) estimated gene flow among species based on FST, however, such estimation can be misleading and should be interpreted cautiously (reviewed by Whitlock and McCauley 1999). In the study

by Devey et al. (2008), based on phylogenetic analysis, the authors suggested that cross-pollination is common among species because no clear phylogenetic patterns were found based on DNA sequences (*ITS* and plastid markers) and AFLP markers. However, gene flow cannot be assessed based on phylogenetic analysis without proper population genetic data (Slatkin 1985). Interestingly, Devey et al. (2008) found no significant genetic differentiation between *O. exaltata* and *O. sphegodes* (based on their *ITS*, plastid markers, and AFLP), whereas we found a clear clustering pattern with AFLP markers and even more so with floral-odor bouquet analyses. This discrepancy is likely because our study provides a more fine-grained resolution through the analysis of multiple populations and large sample sizes. We suggest one should be careful in drawing any conclusions on gene flow from investigations using only molecular markers or morphological data (see also discussion on hybridization in *Ophrys* below). As a consequence of the suggested gene flow across putative species boundaries, and the typically high variability among individuals in *Ophrys*, some authors have lumped several species together, resulting in a classification of few species and many subspecies (Pedersen and Faurholdt 2007). In *Ophrys*, species identification based on morphological characters alone can indeed be difficult. Floral scent, however, often shows a specific pattern among closely related, and morphologically very similar species (Mant et al. 2005b; Stökl et al. 2009), and should thus be taken into consideration when assigning individuals into species categories or testing such assignments. To better understand reproductive isolation and the implicated taxonomic consequences in *Ophrys*, we suggest that a complementary approach should be taken. This approach should incorporate the quantification of floral isolation and later-acting reproductive barriers (Ramsey et al. 2003), and combine these results with the analysis of the traits under selection (e.g., floral odor) as well as neutral molecular markers.

MECHANISMS OF FLORAL ISOLATION IN OPHRYS

In the studied species only ethological isolation contributes to floral isolation, because all three species attach pollinia to their pollinators' heads, and hence there is no evidence of mechanical isolation. In *Ophrys*, floral odor acts as a key trait for specific pollinator attraction (Schiestl et al. 1999; Mant et al. 2005b) and is therefore likely responsible for ethological isolation among species. Our

discriminant function analysis of floral odor bouquets showed a clear separation among each species (Fig. 3 D,E,F). It has been shown that *O. sphegodes* attracts males of *Andrena nigroaenea* by emitting a hydrocarbon mixture with high proportions of (Z)-9 and (Z)-11/12 alkenes (Schiestl et al. 1999) (Fig. S3),whereas *O. exaltata* attracts males of *Colletes cunicularius* (Fig. S4) by emitting high proportions of (Z)-7 alkenes (Mant et al. 2005a). The pollinator of *O. garganica* has been reported to be *Andrena carbonaria* (Paulus and Gack 1990b) (Fig. S5); *O. garganica* emits high proportions of (Z)-9 and (Z)-11/12 alkenes with different carbon chain lengths (typically longer than in *O. sphegodes*), however, the active compounds for its pollinator have not yet been identified. Behavioural tests showed that *C. cunicularius* was only attracted by the floral odor of *O. exaltata*, but not by the other two species (Mant et al. 2005b). Recently, Vereecken and Schiestl (2009) showed that floral color differences between *O. sphegodes* and *O. exaltata* do not contribute to species-specific pollinator attraction. Collectively, these data suggest that strong ethological isolation in *Ophrys* is primarily due to different floral odor bouquets produced by each species, which are linked to the attraction of different, highly specific pollinators.

HYBRIDIZATION AMONG *OPHRYS* SPECIES

Pollinator adaptation may drive floral diversification and speciation in *Ophrys*, however, hybridization has sometimes been considered to be common among *Ophrys* species (Devey et al. 2008). In contrast, putative *Ophrys* F1 hybrids (as identified by morphology) were often found to be solitary, with large number of plants from the parental species surrounding them (Stebbins and Ferlan 1956), suggesting hybridization may not happen frequently. In accordance with this, we found only two putative F1 hybrids among 146 samples (1.37%) of *O. sphegodes* and *O. exaltata*. Both were found in one population (FCG). Those two putative F1 hybrids produced a floral odor bouquet similar to *O. sphegodes*. Possible (nonexclusive) reasons for hybrids found in natural population could be the following: (1) the strength of floral isolation may be variable among populations due to variable specificity in the responses of pollinators to floral odor bouquets; (2) Changes of floral odor in *Ophrys* may happen through occasional changes in scent genes, leading to a break-down of floral isolation. To test the first

possibility, floral isolation should be assessed in various populations. We found consistently strong floral isolation in two adjacent populations, but could not precisely estimate floral isolation in the more distant population FCG, because the total number of pollination events observed in this population was small (only three in total). A study by Vereecken et al. (2010) suggested that floral isolation among *Ophrys* species pollinated by *C. cunicularius* and *Andrena nigroaenea* can break down in some populations, although the frequency of hybrids in that study was always much lower than that of parental species. Break down of floral isolation was also found in some populations among *Ophrys* species pollinated by other *Andrena* species (Stökl et al. 2008; Cortis et al. 2009). Varying strengths of floral isolation would suggest a geographical mosaic, with merging of populations through hybridization in some areas and divergence through strong floral isolation in other areas. This geographic mosaic may help to explain the phylogenetic pattern of *Ophrys* species observed in previous studies (Soliva et al. 2001; Devey et al. 2008). However, further investigations about geographical variation in pollinator behaviour and floral isolation are needed to evaluate this hypothesis. A second reason for hybrids occasionally found in nature may be the genetic basis of floral odor changes in *Ophrys*. Because changes in floral odor production in *Ophrys* may be brought about by few genetic changes (Schlüter and Schiestl 2008, Schlüter et al. 2011), one would expect that some individuals of one species could stochastically evolve the same floral odor as another species through mutation or recombination. This would eventually lead to hybridization in natural populations, considering that floral odor is the major attractant for specific pollinators in this system and postpollination barriers are effectively absent. This hypothesis is consistent with the genetic and floral odor analyses in this study. Among the samples investigated here, a few plant individuals showed mismatches among their assignments from genetic and odor data (samples S07, G10, G14, S47 in Fig. 3, summary in Table 4). In other words, these samples have the neutral genetic background of one species, but an odor phenotype of another species, possibly due to changes in few genes controlling floral odor production. However, to further test this hypothesis, detailed studies on the genetic basis of floral odor components in *Ophrys*, and their consequence for pollinator attraction are needed.

POLLINATOR ADAPTATION AND SPECIATION IN *OPHRYS*

As a scenario for speciation in *Ophrys*, we propose that incipient *Ophrys* species adapt to different pollinators by changing floral traits, especially floral odor, that convey strong floral isolation and induce the speciation process. In *Ophrys*, pollination success is relatively low due to pollen limitation (compare pollination success of natural populations and fruit set rate from hand pollination); a sexually deceptive pollination mechanism may thus induce negative density-dependent selection: high population density may lead to low pollination success because pollinators are more likely to learn and avoid the deceptive flowers. Therefore, a shift in pollinators mediated by a change in floral scent genes may convey a selective advantage by increasing pollination success in the initially few novel genotypes. Furthermore, as shown in our study, different pollinators in *Ophrys* are associated with strong floral isolation, which is sufficient to prevent significant gene flow in sympatry. Changes of floral odor bouquets may be based on changes in few genes involved in the biosynthesis (or regulation) of pollinator-attractive floral compounds (Schlüter and Schiestl 2008; Schlüter et al. 2011). Therefore, speciation *in Ophrys* could happen rapidly, even in sympatry. For example, Vereecken et al. (2010) showed that novel floral odor bouquets in *Ophrys* could evolve rapidly (after only one generation of hybridization), and directly lead to pollinator shifts in sympatry. The remarkable plant–pollinator interaction in *Ophrys* orchids provides a particularly interesting system to study pollinator adaptation directly involved in species divergence, a process that may be important in several other, highly specific pollination systems (Schiestl, in press).

CONCLUSIONS

By in situ tracking pollen flow and experimental hand pollination, we found floral isolation to be very strong among closely related, sympatric *Ophrys* species, whereas later-acting barriers to gene flow were effectively absent. Our results provide direct evidence that the reproductive barrier among these closely related plant species with specialized pollination consists mostly of floral isolation. In such a system, pollinator adaptation could directly lead to floral isolation and

speciation. This offers a particular opportunity to study the role of floral isolation during the evolution of reproductive isolation and speciation. However, further studies that systematically combine neutral traits (such as molecular markers), traits under selection (such as floral odor) and their genetic basis, pollinator behaviour, as well as quantification of floral isolation in natural habitats will be helpful to better understand speciation processes in plants with specialized pollination systems.

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SUPPORTING INFORMATION

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The following supporting information is available for this article:

Figure S1. Observation error rate estimation and simulated floral isolation index among species.
Figure S2. Ploidy levels of the three Ophrys species
Figure S3. Ophyrs sphegodes and its pollinator Andrena nigroaenea.
Figure S4. Ophyrs exaltata and its pollinator Colletes cunicularius.
Figure S5. Ophyrs garganica and its pollinator Andrena carbonaria.

"SPECIATION BY DISTURBANCE: A POPULATION STUDY OF CENTRAL ITALIAN *OPHRYS SPHEGODES* LINEAGES"

INTRODUCTION

Speciation by selection can be the result of different fixed mutations contributing to adaptation, or it is caused by environmental pressures – the latter process is known as ecological speciation and has been to shown to be a common force driving divergence in parapatry (Schluter 2009). While the basic idea dates back to Mary's evolutionary synthesis (1942), the subject recently gained an intensified interest (e.g. Schluter 2009). Ecological speciation can be defined "as the process in which barriers to gene-flow evolve between populations as the result of ecologically-based divergent selection" (Rundle & Nosil 2005).

The mechanisms that drive divergence in allopatry are well studied. Allopatric speciation involves the simple and easy to proof concept that divergence from a common ancestor is due to genetic drift and differential selection pressures over time in spatial isolation. While the latter scenario forbids gene-flow, parapatric or sympatric speciation necessarily include genetic exchange at least in the early steps of divergence. However, we are left with the question what the factors are that drive diversification in adjacent populations. This is of special interest in saturated plant communities where the ecological niche space is occupied in most parts.

The framework of speciation by disturbance as a sub-concept of ecological speciation provides us with an explanation how saturated plant communities can be invaded by new species, or how it can promote lineage divergence. Long-term or periodic disturbance in the form of climate changes, pathogens, and most of all human intrusions leads to the alteration of existing niches and the emergence of new ones. The inherent concept implies that human colonisations may not only have led to a decrease of biodiversity through deforestation, ground sealing and construction activities of any kind. It also will have promoted the diversification of lineages into the new disturbed habitats. As evolution is happening very slowly from the human perspective, there might have been insufficient time for the incipiently diverging lineages to accumulate genetic

differentiation that allows detection with genetic tools or phenetic approaches. The tempo of speciation is a function of the quality of acting divergent selection pressures and the characteristics of the plant system they act on. Therefore, highly different mutation rates are expected to mark different situations and organisms. Fast evolving plant lineages might allow us to uncover the footprints of recent speciation events.

The genus Ophrys is thought to have undergone a rapid radiation, which is likely to be recent as genetic differentiation within closely related taxa is very low. Floral isolation represents probably the only reproductive isolation barrier among species of that genus. Isolation can be achieved by minor alterations of the scent bouquet responsible for attraction of specific pollinators. The switch to a novel pollinator might be based on minor genic changes due to random mutation, drift, or even positive selection. The inherent potential to immediately achieve reproductive isolation makes Ophrys a potential candidate for fast evolution and lineage diversification. As also the respective pollinator community is influenced by disturbance, the dynamics of this pollinator-plant system are probably more eminent than in most other systems. Among others, ecological speciation driven by disturbance is one possible scenario under which lineages of the genus Ophrys could have evolved.

We present an example of a taxon that is likely to have speciated under the influence of human disturbance, as a case study for the whole genus, in which most taxa grow in disturbed habitats.

MATERIALS & METHODS

STUDY SPECIES & POPULATIONS

We studied six populations of the polytypic and widely distributed *O. sphegodes* MILLER in Central Italy with AFLP's. (1) *O. sphegodes s.l.* MILLER, Vesuvio, Campania; (2) *O. sphegodes s.l.* MILLER, Cuma, Campania; (3) *O. sphegodes s.l.* MILLER, Foce Garigliano, Campania; (4) *O. sphegodes s.str.* MILLER, Gargano, Puglia; (5) *O. argentaria* J. DEVILLERS-TERSCHUREN & P. DEVILLERS (syn. *O. sphegodes ssp. litigiosa var. argentaria* (J. DEVILLERS-TERSCHUREN & P. DEVILLERS) N. FAURHOLDT), Marina di Castagneto, Tuscany; (6) *O. classica* J.

DEVILLERS-TERSCHUREN & P. DEVILLERS (*O. sphegodes* auct. non. MILLER), Marina di Castagneto, Tuscany. Scent was analyzed for populations (2) and (4), and additionally for (7) *O. exaltata s.l.* (syn. *O. exaltata ssp. archipelagi*), Cuma, Campania; (8) *O. exaltata s.l.* (syn. *O. exaltata ssp. archipelagi*), Gargano, Puglia. The same latter four populations were also investigated with pollinator choice experiments.

AMPLIFIED FRAGMENT LENGTH POLYMORPHISM METHOD (AFLP)

The amplified fragment length polymorphism procedure was performed as described by Vos et al. (1995), with modifications as reported in Moccia et al. (2007) and using fluorescent dye-labeled primers. An initial trial using 14 different primer combinations on four individuals each of *O. sphegodes* and *O. exaltata* was conducted to identify those primers that yield the highest number of easily scorable peaks that were different between the two species. After the screening six primer combinations were chosen: AGCfamACAC, ACCnedACTG, AGChexATCG, ATGfamCGG, AACnedCGC and AGChexCCAA. For the restriction digestion the enzymes EcoRI and MseI were used on a total of 250 ng DNA. Ligation of EcoRI and MseI adapters took place in the same reaction. 2µl of the restriction-ligation product was used for a preselective PCR with EcoRI and MseI, both having one additional selective base. For the successive selective PCR, 1µl of a 1:10 dilution of the PCR product was used. Primers were the same as in the preselective PCR, but with three or four additional selective bases. Fragment separation and detection took place on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, USA). 500 LIZ was used as internal standard. Alignment of the raw data and detection of the fragment sizes were done with Genemapper 3.7 software (Applied Biosystems, Foster City, USA). Absence or presence of AFLP bands were carefully scored by eye. To avoid artefacts only AFLP markers that could be unambiguously scored over the whole data set were included in the binary matrix. AFLP analysis was performed as two experiments at different dates and runs, and scored independently, preventing us from merging the two AFLP datasets. These two separate datasets were therefore analyzed separately: the first dataset from 2009 contains 51 samples: 9 *O. argentaria* (Tuscany TUS), 12 *O. classica* (Tuscany TUS), 15 *O. sphegodes* (Gargano GAR), 15 *O. sphegodes* (Foce Garigliano FCG). The second data set from 2011 contains 95 samples: 17

O. sphegodes (Vesuvio VES), 22 *O. sphegodes* (Cuma CUM), 18 *O. sphegodes* (Foce Garigliano FCG), 18 *O. sphegodes* (Gargano GAR), 9 *O. argentaria* (Tuscany TUS), 11 *O. classica* (Tuscany TUS).

FLORAL ODOR ANALYSIS

GC analysis was performed as described by Mant et al. (2005a) with 300 ng n-octadecane (C18) added to the floral extracts as an IS. One micro liter of each sample was injected into an Agilent 6890 GC at 50◦C, followed by opening of the split valve and heating to 300◦C at rate of 4◦C/min. An HP-5 column and flame ionization detector (FID) were used, and hydrogen was used as a carrier gas, with nitrogen as the makeup gas. For identification of compounds, several samples were re-analyzed by GC with a mass selective detector (GC/MSD; Agilent 5975) using the same oven and column parameters. Spectrum and retention time of compounds were compared with those of synthetic standards, that is, alkanes: nonadecane (C19), henicosane (C21), docosane (C22), tricosane (C23), tetracosane (C24), pentacosane (C25), hexacosane (C26), heptacosane (C27), octacosane (C28), nonacosane (C29); and alkenes: (Z)-7-heneicosene [(Z)-7-C21], (Z)-9-heneicosene [(Z)-9-C21], (Z)-7-tricosene [(Z)-7-C23], (Z)-9-tricosene [(Z)-9-C23], (Z)-7- pentacosene [(Z)-7-C25], (Z)-9-pentacosene [(Z)-9-C25], (Z)- 11-pentacosene [(Z)-11-C25], (Z)-12-pentacosene [(Z)-12-C25], (Z)-7-heptacosene [(Z)-7-C27], (Z)-9-heptacosene [(Z)-9-C27], (Z)-11-heptacosene [(Z)-11-C27], (Z)-12-heptacosene [(Z)-12-C27], (Z)-7-nonacosene [(Z)-7-C29], (Z)-9-nonacosene [(Z)-9- C29], (Z)-11-nonacosene [(Z)-11-C29], (Z)-12-nonacosene [(Z)- 12-C29], where (Z)-number indicates the cis double-bond position. For sources of standard compounds see Mant et al. (2005a). It is noted that the discrimination of (Z)-11- and (Z)-12-alkenes was not possible with the GC parameters used. The relative amount of each odor compound was calculated as the proportion of the total amount of all alkenes and alkanes of a chain length between 18 and 30 carbons.

DATA ANALYSES

The resulting binary matrices from the AFLP scorings were cleaned from columns where a marker could only be scored once over the whole data set. GenAlEx

Fig.1 – Investigated species and populations. upper left: *O. sphegodes s.l.* MILLER, Vesuvio, Campania; u.m.: (2) *O. sphegodes s.l.* MILLER, Cuma, Campania; u.r.: (3) *O. sphegodes s.l.* MILLER, Foce Garigliano, Campania; m.l.: (4) *O. sphegodes s.str.* MILLER, Gargano, Puglia; m.m.: (5) *O. argentaria* J. DEVILLERS-TERSCHUREN & P. DEVILLERS (syn. *O. sphegodes ssp. litigiosa var. argentaria* (J. DEVILLERS-TERSCHUREN & P. DEVILLERS) N. FAURHOLDT), Marina di Castagneto, Tuscany; m.r.: (6) *O. classica* J. DEVILLERS-TERSCHUREN & P. DEVILLERS (*O. sphegodes* auct. non. MILLER), Marina di Castagneto, Tuscany; l.l.: (7) *O. exaltata s.l.* (syn. *O. exaltata ssp. archipelagi*), Cuma, Campania; l.m.: (8) *O. exaltata s.l.* (syn. *O. exaltata ssp. archipelagi*), Gargano, Puglia; l.r.: Pseudo-copulation of *Colletes cunicularius* on *O. exaltata*, Marina di Lesina, Gargano, Puglia, It.

(Peakall & Smouse 2006) was used as macro in Microsoft Excel to calculate genetic distances as basis for generating PCoA graphics. FAMD (Schlüter & Harris 2006) was used to calculate genetic distances, pairwise □-ST values and to generate NJ population trees. Linear discrimination analysis (LDA) in R was used for analysis of floral scent based on relative amounts of hydrocarbons.

POLLINATOR CHOICE EXPERIMENTS

Fidelity of *Ophrys* pollinators was investigated in an experimental plot design. Plant material was collected in natural populations along the Tyrrhenian (Cuma, Vesuvio) and Adriatic Coast (Capoiale, Marina di Lesina) where both, *O. sphegodes* and *O. exaltata ssp. archipelagi* occur sympatrically. Plots with plants of both species from the two coasts were installed in Cuma and the Gargano region. Each plot consisted of four plants, with one individual of each of the two species from both coasts. The inflorescences were placed in a random order with 30 cm distance between them in flowering bushes along sandy paths in the Macchia vegetation. Male bees are patrolling along sandy foot paths where many nesting places of female solitary bees have been observed; the male bees also fly up to check for females in flowering bushes (*Rosmarinus officinalis*, *Spartium junceum*, *Hippocrepis sp.*), as the female bees are used to forage there on nectar and pollen. Plants were exchanged every 30 minutes, regardless of their attractivity to pollinators. Pollination events were recorded only when the bee was successfully caught after an observed pseudocopulation with pollinia removal. The bees were later identified by comparison to a reference collection of *Ophrys* pollinators at the University of Zürich, Switzerland; in many cases the genitals of the male bees had to be preparated and checked under the microscope. Additionally we caught and identified the pollinators of the *O. sphegodes* population on Vesuvio (CA, It.) and of the *O. fusca s.l.* population in Gargano, which occurs sympatric with *O. sphegodes* and *O. exaltata*.

FLORAL ISOLATION PLOTS

An experimental approach with a plot design was used to measure floral isolation. The plots were set up in the same location as naturally occurring plants. One individual of each species were randomly positioned in each plot (each plot

contained one specimen each of *O. sphegodes* (Gargano), *O. sphegodes* (Cuma), and *O. fusca s.l.* (Gargano)). The distance between neighbouring plants was 0.5 m. For each experiment, 20 plots were set up along a transect through the habitat of the orchids. The distance between neighbouring plots was 20 m, since the average pollinia carrying distance of *Colletes cunicularius* has been estimated to be around 5 m (Peakall and Schiestl 2004). Plants for the plot experiments were picked from natural populations. Each flower was checked for pollinia removal or pollen deposition. Pollinia have been stained alternately with the dyes brilliant green, methylene aniline blue, orange G and trypan red as described in Peakall et al. (1989). The colors used for each species were randomized between experiments to reduce potential effects of staining color on pollinator behaviour. The inflorescence was put in a water-filled 15 ml plastic tube placed into the ground. Pollinia removal and deposition on stigmas were recorded three days after setting up the plots.

RESULTS

POLLINATORS

We set up 10 choice-plots on 8 days in the period of 17/03 – 31/03/2011 for (2—) 3 (—4) hours each, in the morning hours from 9am until 1pm. The four studied populations co-flower at the end of March/beginning of April. Though, it has to be noted that the *O. sphegodes s.l.* population in Cuma starts to flower as early as end of January, while the three other populations begin flowering about one month later. Three plots were installed in Cuma (9 hours), seven plots in Gargano (19 hours). Pollinator activity is negligible in the afternoon, and overall activity in Gargano was notably higher than in Cuma. A summary of caught pollinators is given in Tab.1. *O. exaltata* GAR attracted 4 times *C. cunicularius*, *O. sphegodes* GAR 3 times *A. nigroaenea*; i.e. both taxa from Gargano attracted their legitimate pollinators on both sides of the Italian Peninsula. We found a different situation for the two taxa from Cuma: *O. sphegodes* CUM attracted 6 times *C. cunicularius*, but also 2 times a yet unidentified bee of the genus *Eucera*. This was found only in the Cuma population, and it's the first time that a *Eucera* bee is reported to pollinate a taxon from the *O. sphegodes* group. *O. sphegodes*

CUM attracted 4 times *A. nigroaenea*, but 49 times *A. bimaculata*. Outside of the choice-plots we confirmed that *O. sphegodes* from Vesuvio attracts also *A. bimaculata*, which was caught several times on plants from this population. This bee species has been reported to pollinate some East-Mediterranean taxa, but has not been reported for any Central- to West-Mediterranean species yet. *A. bimaculata* (2x) *A. nigroaenea* (1x) and *C. cunicularius* (2x) were found to pollinate *Ophrys fusca s.l.*.

Tab.1 – Pollinator choice experiment: Bee counts. CUM=Cuma, Napoli, Tyrrhenian Coast, It., GAR=Gargano, Marina di Lesina/Capoiale, Adriatic Coast, It. The *Eucera* taxon is yet un-identified. *=yet unreported pollinator species. #=*Eucera sp.* was only caught in Cuma, while all other pollinator species were observed to visit the same species on both coasts.

	Andrena bimaculata	***Andrena nigroaenea***	***Colletes cunicularius***	***Eucera sp.***
***O. sphegodes* CUM**	49 *	4		
***O. sphegodes* GAR**		3		
***O. exaltata* CUM**			6	2 *#
***O. exaltata* GAR**			4	

GENETIC STRUCTURE

Genetic differentiation between the investigated populations is generally low. The first analysis with four populations yielded 322 variable markers. The resolution is slightly better than in the second analysis of six populations, where only 148 markers could unambiguously be scored. The three populations from the Tyrrhenian Coast stay close together in the PCoA, but they largely overlap with the populations from Tuscany and Gargano. Of the Tyrrhenian populations, FCG and CUM are most distant to the Adriatic GAR population, while VES is most close to GAR (Fig.3a). The two populations from Tuscany largely overlap with each other and take in an intermediate position between the CUM/FCG populations and the GAR population (Fig.3b). Analysis of the small dataset revealed two possible hybrids: one sample of GAR clusters with the Tuscany populations, another sample of ARG-TUS appears far away from all other populations. Population NJ trees show a good separation of populations in the small dataset,

but strong admixture in the large dataset; this is due to technical problems experienced in the fragment detection on the sequencer. The tree generated from the small dataset displays SPH-GAR together with some accessions of SPH-FCG and CLA-TUS as sister to SPH-FCG, the positions of ARG-TUS and CLA-TUS are unresolved in a polytomy.

Tab.2 – Pairwise Φ_ST values (Coefficient: Standard Jacquard. Distance Transformation: d=1-s) for the 95 ind./6 pops. AFLP dataset. Highest and lowest values colored, in italics.

SPH-FCG	SPH-CUM	SPH-VES	SPH-GAR	ARG-TUS	CLA-TUS
0.0000000000					
0.0102326582	0.0000000000				
0.0644884038	0.0504072577	0.0000000000			
0.2511072708	0.2750169381	0.1431484848	0.0000000000		
0.3077216546	*0.3484977846*	0.3003501691	0.3171436351	0.0000000000	
0.0995553941	0.1742699753	0.0898278018	0.1828787903	0.1111831264	0.0000000000

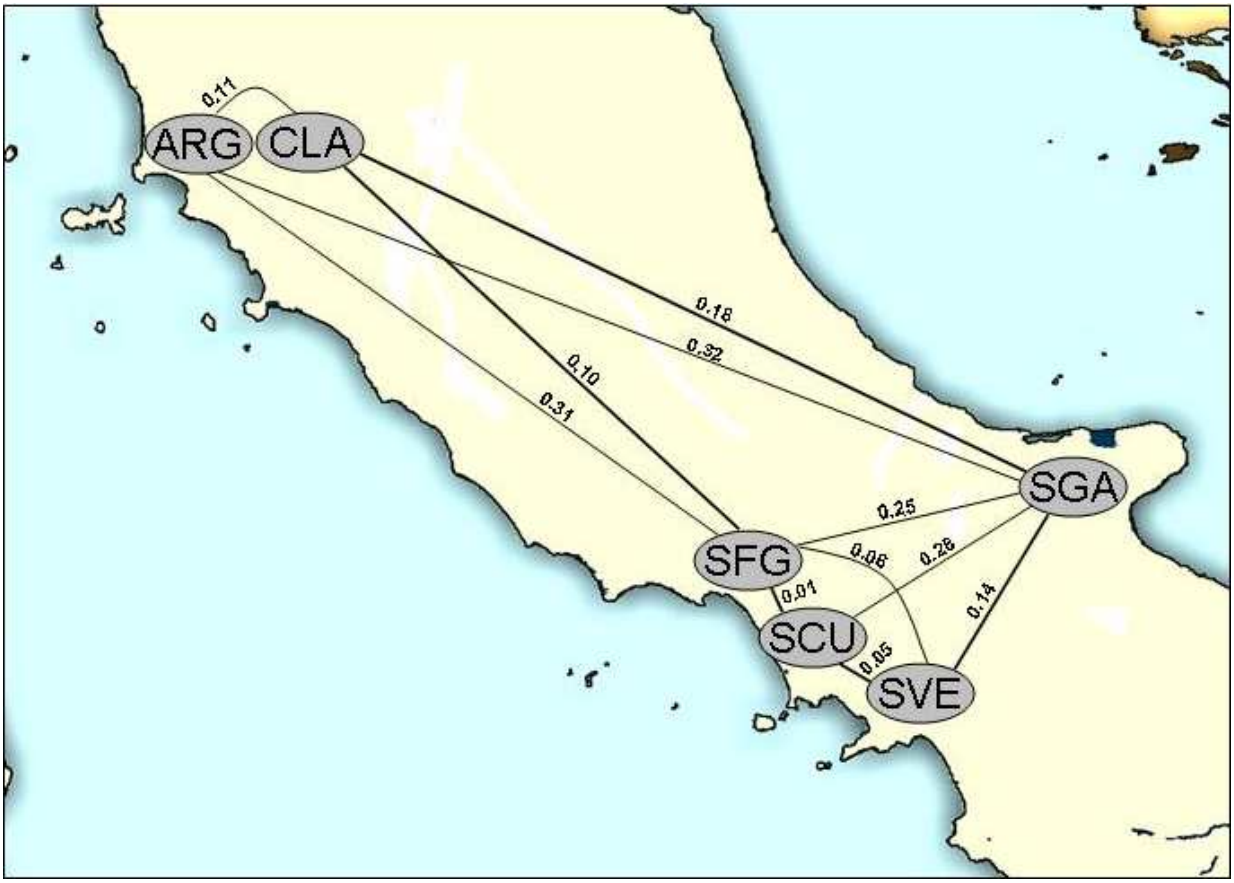

Fig.2 – Genetic divergence. Pairwise Φ_ST values. SVE=*O. sphegodes*, Vesuvio, CA; SCU=*O.sphegodes*, Cuma, CA; SFG=*O. sphegodes*, Foce Garigliano, CA; CLA=*O. classica*, Marina di Castagneto, TU; ARG=*O. argentaria*, Marina di Castagneto, TU; SGA=*O. sphegodes*, Gargano, PU;

Genetic divergence between population pairs in the large dataset was assessed with Φ_ST value comparisons (Tab.2). Lowest Φ_ST values were found between SPH-FCG and CLA-TUS (0.01), highest between SPH-CUM and CLA-TUS (0.35). Interestingly, genetic divergence between SPH-VES and the distant SPH-GAR is lower than between SPH-VES and the spatially close SPH-CUM/SPH-FCG (Tab.2, Fig.2)

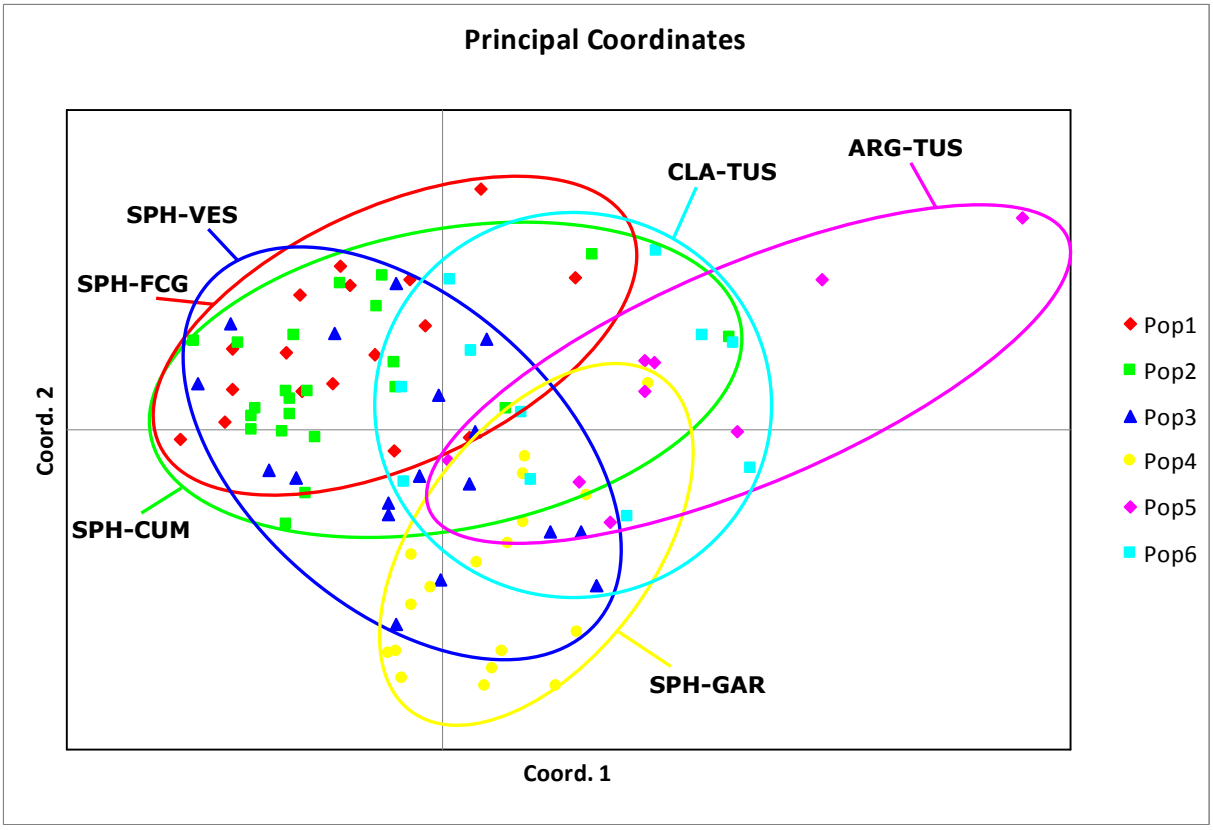

Fig.3.a – Genetic structure of Italian *O. sphegodes* group taxa. PCoA from AFLP fingerprinting data 148 markers of 95 individuals from six populations: *O. sphegodes s.l.* from Vesuvio, CA (SPH-VES), *O. sphegodes s.l.* from Cuma, CA (SPH-CUM), *O. sphegodes s.l.* from Foce Garigliano, CA (SPH-FCG), *O. sphegodes s.str.* from Gargano, PU (SPH-GAR), *O. argentaria* from Marina di Castagneto, TUS (ARG-TUS), *O. classica* from Marina di Castagneto, TUS (CLA-TUS). The first two axes explaining 25.5 and 19.4 percent of variation, respectively.

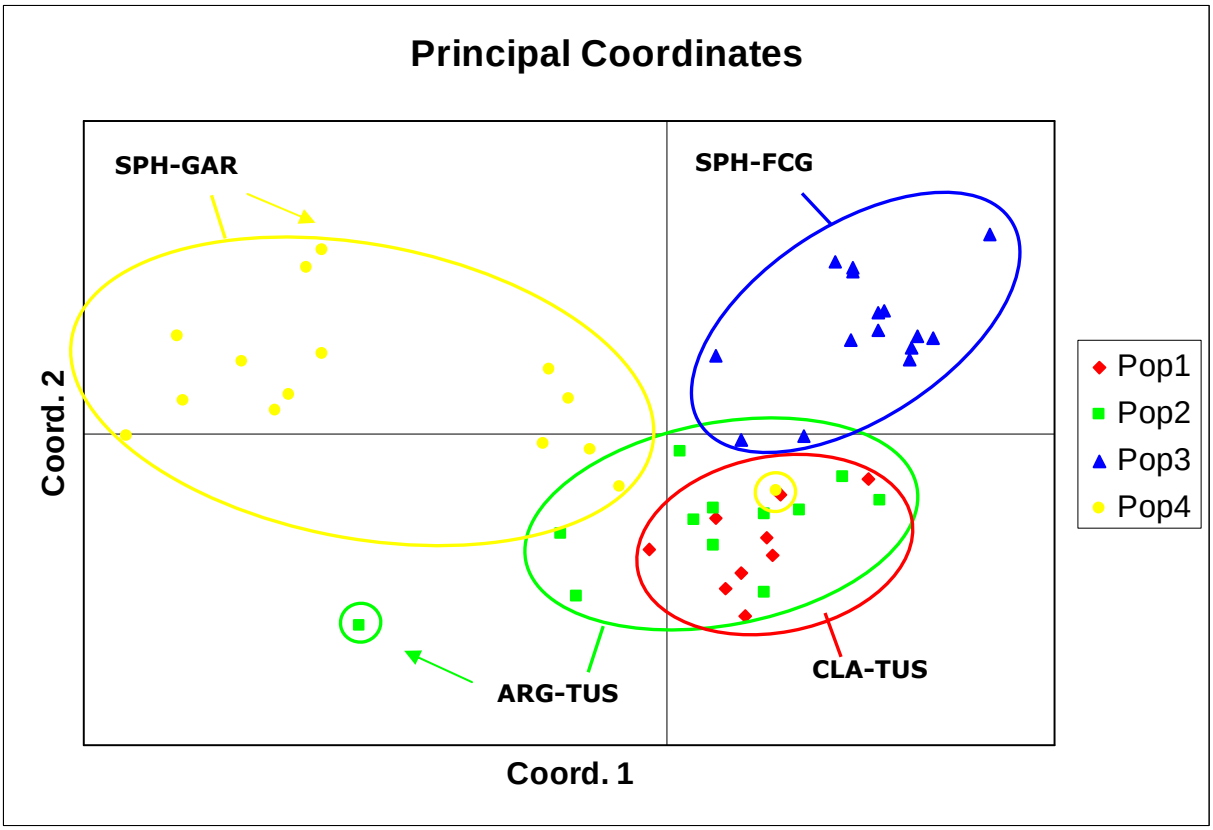

Fig.3.b – Genetic structure of Italian *O. sphegodes* group taxa. PCoA from AFLP fingerprinting data of 405 markers of 51 individuals from four populations: *O. sphegodes s.l.* from Foce Garigliano, CA (SPH-FCG), *O. sphegodes s.str.* from Gargano, PU (SPH-GAR), *O. argentaria* from Marina di Castagneto, TUS (ARG-TUS), *O. classica* from Marina di Castagneto, TUS (CLA-TUS). The first two axes explaining 23.5 and 17.9 percent of variation, respectively.

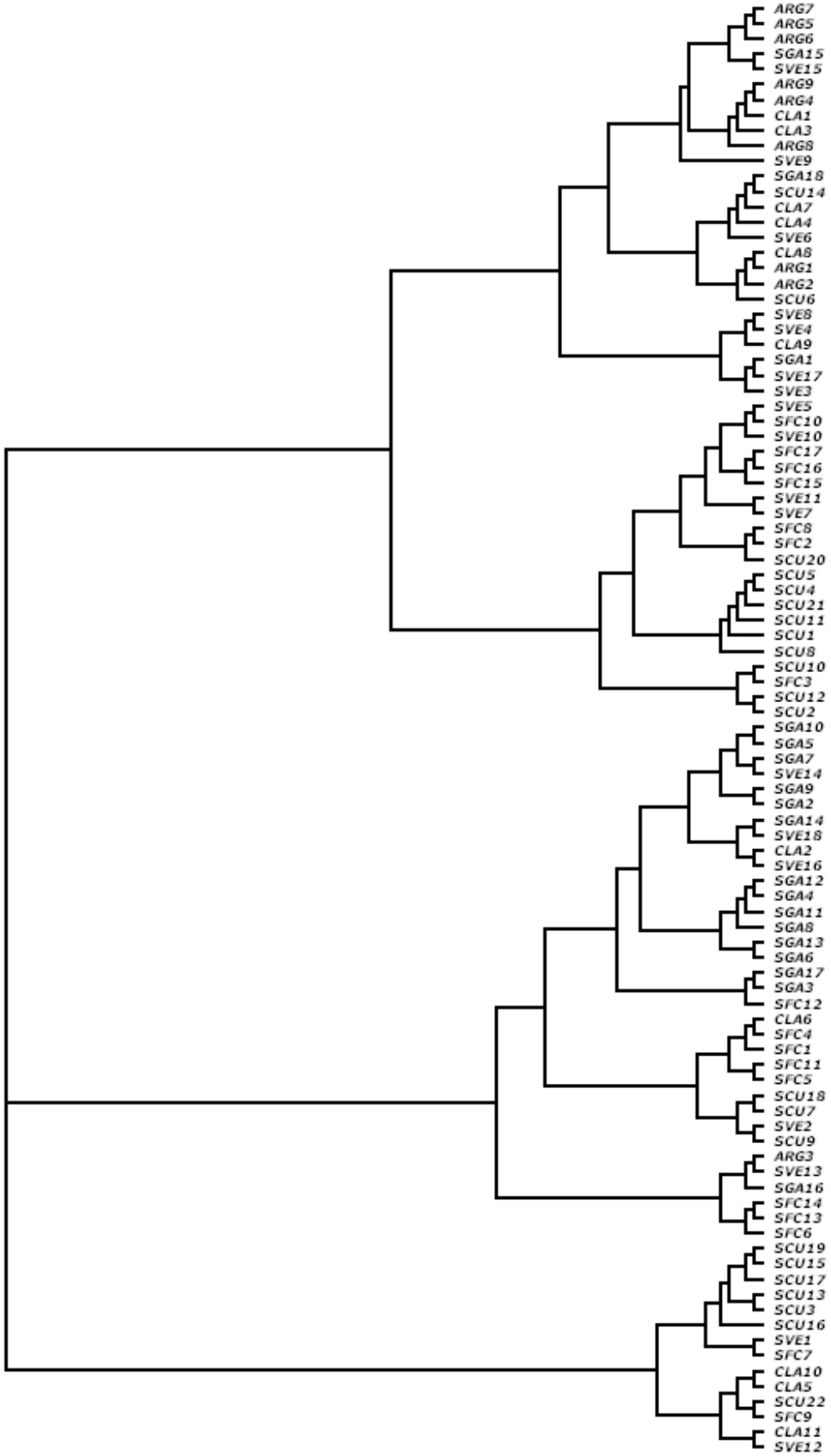

Fig.4 – NJ tree for the 95 ind./6 pops. AFLP dataset (standard Jacquard's similarity). SVE=*O. sphegodes*, Vesuvio, CA; SCU=*O.sphegodes*, Cuma, CA; SFG=*O. sphegodes*, Foce Garigliano, CA; CLA=*O. classica*, Marina di Castagneto, TU; ARG=*O. argentaria*, Marina di Castagneto, TU; SGA=*O. sphegodes*, Gargano, PU;

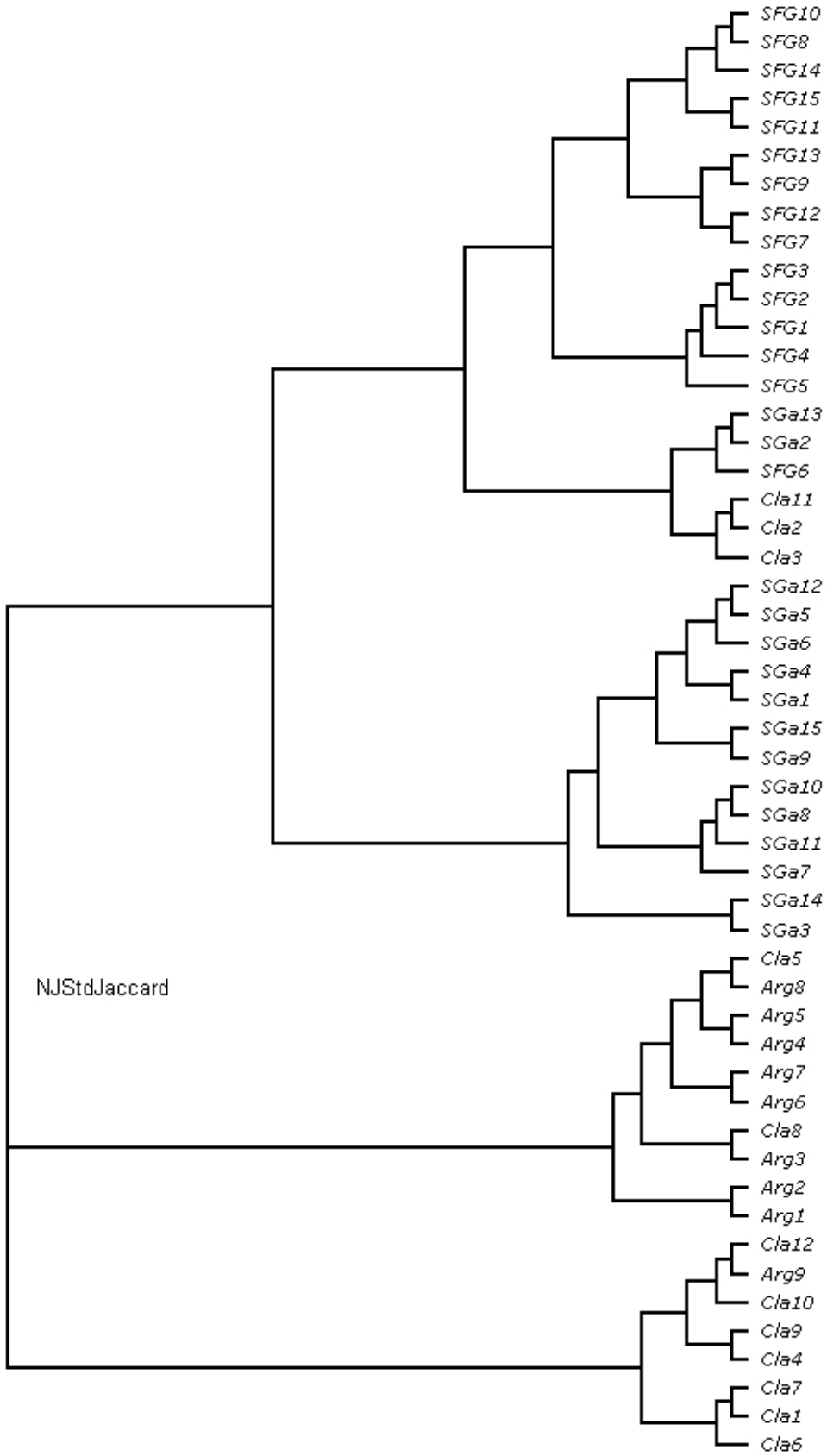

Fig.5 – NJ tree for the 51 ind./4 pops. AFLP dataset (standard Jacquard's similarity). SFG=*O. sphegodes*, Foce Garigliano, CA; CLA=*O. classica*, Marina di Castagneto, TU; ARG=*O. argentaria*, Marina di Castagneto, TU; SGA=*O. sphegodes*, Gargano, PU;

FLORAL ODOR

The linear discrimination analysis plot of *O. exaltata* and *O. sphegodes* from the opposite Tyrrhenian and Adriatic Coast shows a slight separation between the *O. exaltata* populations, and moderate separation with partial overlap between the two *O. sphegodes* populations. An analysis of physiologically active scent compounds revealed the same semio-chemicals in similar proportions in both populations of *O. exaltata*. The comparison between the *O. sphegodes* populations reveals significant differences in the relative proportions of scent components in floral extracts. Two components are exclusive for the SPH-CUM population: C23.Z7 and C27.Z7.

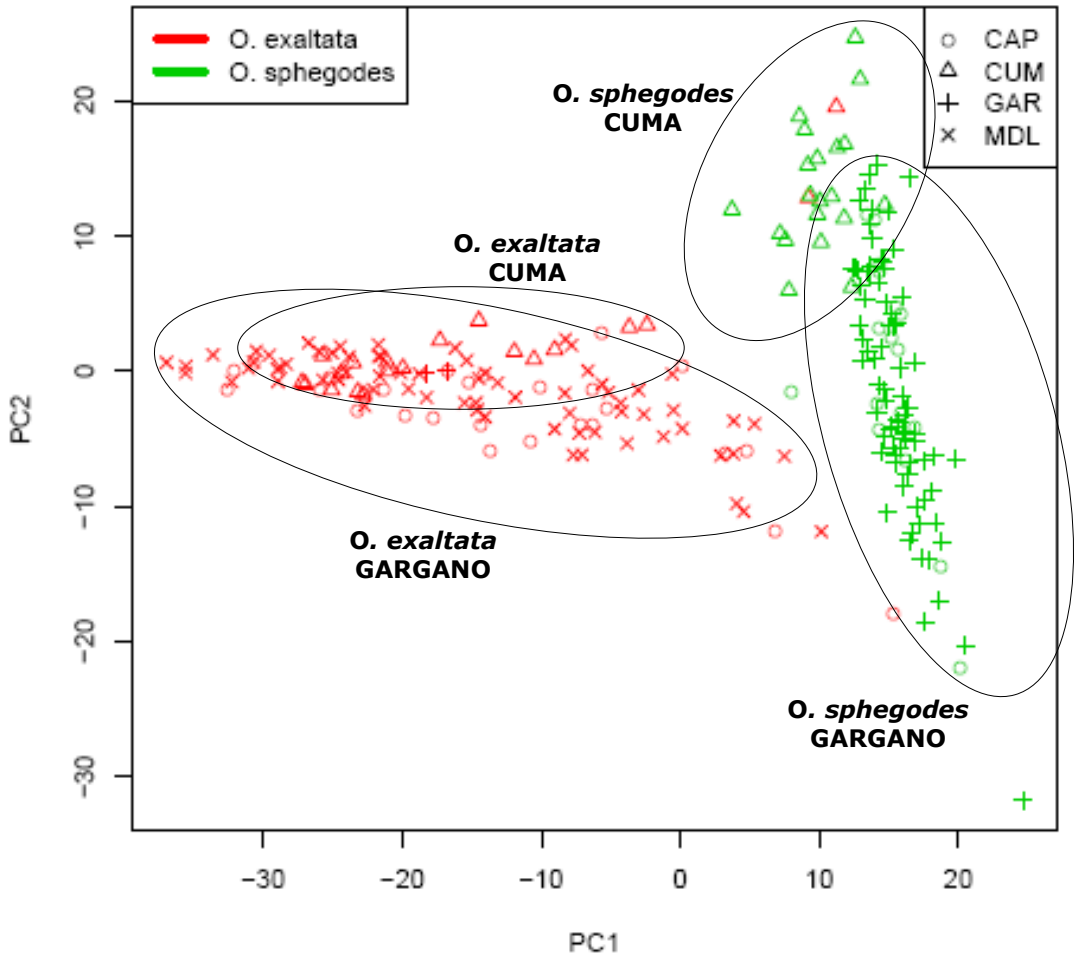

Fig.6 – Linear discrimination analysis (LDA) of floral scent from Italian populations of *O. sphegodes s.l. and O. exaltata s.l.* CAP – Capoiale, PU, CUM – Cuma, CA, GAR – Gargano, PU, MDL – Marina di Lesina PU.

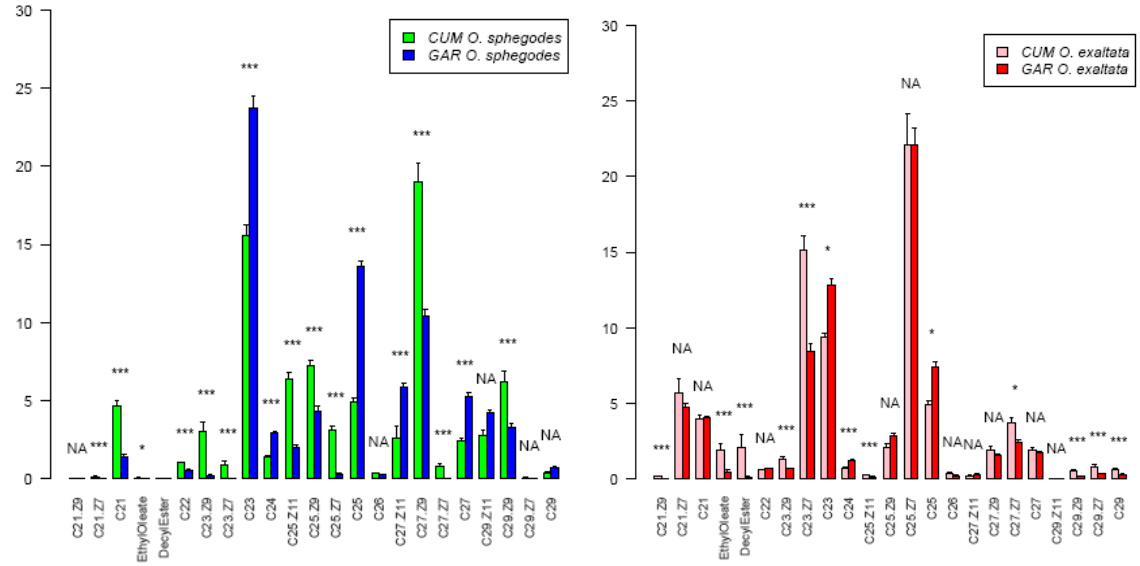

Fig.7 – Scent profiles of Italian populations of *O. sphegodes s.l.* and *O. exaltata s.l.* CUM – Cuma, CA, GAR – Gargano. x-axis: carbon hydrates and one ester, y-axis:

FLORAL ISOLATION

As we didn't record a single transfer of stained pollen this approach was discarded. Besides some pollen removals the experiment didn't yield any results. As the plants were placed in the natural populations, we assume a general low pollinator activity or climatic phenomena to be responsible.

DISKUSSION

Choice experiments with the two putative *O sphegodes* taxa and another sympatrically occurring *Ophrys* species (*O. archipelagi*) in two distant populations along the Tyrrhenian and Adriatic coasts of Italy revealed the putative *O. sphegodes* from Naples (incl. Vesuvio and Foce Garigliano) as yet undiscovered ecological species: its main pollinator was identified as *Andrena bimaculata*, while the pollinator of *O. sphegodes* from the Adriatic Coast was also responsible for about 12% of overall pollinia removal (Tab.1). Floral isolation between was found to act as an effective isolation barrier between *O. sphegodes* and *O. exaltata* on both coasts, as no pollinator caught indicated gene-flow. Lowest genetic divergence was detected between the *O. sphegodes* populations along the Campanian coast. No pollinators were caught in the FCG population, but due

to the bare genetic differentiation between SPH-CUM and SPH-FCG (Φ_ST = 0.01), the morphological similarity and the shared coastal habitat, it can be assumed that *A. bimaculata* is the local pollinator in FCG. The Adriatic *O. sphegodes* are not strongly diverged from all Tyrrhenian populations, they are genetically most close to CLA-TUS and SPH-VES.

Results of a GC-analysis of floral scent from *O. archipelagi* and the *O. sphegodes*-like taxa from both coasts showed stronger differentiation between the *O. sphegodes*-like taxa, than between *O. archipelagi* from the two distant populations (Fig.6). Two active compounds found (C23.Z7 and C27.Z7) are exclusive to the *O. sphegodes*-like taxon from Napoli (Fig.7). These components might play a key role in the attraction of *A. bimaculata*. Interestingly, *A. nigroaenea* is still attracted by the scent of the Tyrrhenian plants, even though the composition is quite different from the Adriatic plants.

Fig.8 – Leaky Mechanical Isolation 1 (Intersectional hybrid → sect. Pseudophrys x Euophrys). (middle; picture: Marina di Lesina, 2009)**, l**ikely between *O. fusca s.l.* (left; picture: Marina di Lesina, 2011) and *O. exaltata s.l.* (right; picture: Marina di Lesina, 2009). Both species (l. r) share *Colletes cunicularius* as pollinator.

The Napolitan taxon might be the result of a local adaptation of the *Andrena nigroaenea* pollinated *O. sphegodes* to a different pollinator, though with incomplete reproductive isolation when occurring in sympatry. But all investigated populations in Campania are disjunct, and the rarely co-occurring *O. apifera*, *O. exaltata* and *O. bombyliflora* are pollinated by rather different *Eucera* and *Colletes* species.

The identification on *A. nigroaenea*, *A. bimaculata*, and *C. cunicularius* as pollinators of different plants of the local *O. fusca s.l.* variant could be due to

divergent odour phenotypes in a sub-structured population attracting different pollinator species. Or this taxon is sufficiently isolated through mechanical isolation (i.e. differing pollinia placement on the pollinator's body) from sympatric *Ophrys* species they share a pollinator with. In this case there is pollinator sharing with *O. exaltata* (*C. cunicularius*) (Fig.8) and *O. sphegodes* (*A. nigroaenea*) (Fig.9). In fact we found Hybrids that are clearly the result of a cross between an *O. fusca s.l.* and an Eu-Ophrys species (Fig.8). While the local *O. fusca s.l.* likely represents one progenitor, some morphological characteristics point to *O. exaltata* as the other progenitor: reddish coloration on the labellum and a yellow median appendix on the most distal point of the labellum (Fig.8).

Fig.9 – Leaky Mechanical Isolation 2 / Cuma *Ophrys* pollinators. left: *A. nigroaenea* on *O. fusca s.l.* and *O. sphegodes* (both: Marina di Lesina, Gargano, PU, It. 2009). right: *A. bimaculata* on *O. sphegodes s.l.*, and *C. cunicularius* on *O. exaltata* (both: Cuma, CA, It., 2010).

The existence of hybrid zones of secondary contact with the Adriatic *O. sphegodes*, or a west-east stretched continuum seems plausible, but zones of sympatry of the two differentially pollinated lineages have not been found so far. Interestingly, all in-deep pollinator studies of *Ophrys* taxa revealed at least one other, minor pollinator, some of the findings indicating gene-flow with other sympatric *Ophrys* species. But in the absence of other *Ophrys* species and pollinator sharing, gene-flow, and/or the reinforcement of reproductive isolation barriers are unlikely scenarios. Therefore, also genetic drift could have played a role in the evolution of the floral scents' attractiveness to a novel pollinator. Not necessarily excluding the drift scenario, the predominance of *A. bimaculata* over *A. nigroaenea* as pollinators can also be seen as the driving force of selection (density-dependent selection). Spatial absence of *A. nigroaenea* or quantitative dominance of *A. bimaculata* would have had caused drift, or imposed a positive

selection pressure on traits favouring the attraction of the latter pollinating bee species, respectively.

SPECIATION BY DISTURBANCE

It has been shown that long-term or repeated disturbance of the environment are a pre-requisite for invasive plant species to enter previously saturated plant communities (Hobbs 1989). Manmade intrusions contribute to the subdivision of the ecological niche-space (Levin 2004), therefore making it more species-rich.
The coastal vegetation of Campania, which is in parts protected in the Parco Regionale Campi Flegrei and particularly the Riserva Naturale Foce Volturno e Costa Di Licola in the province of Naples, Campania, Italy, is such a disturbed area. Beaches, followed by dunes, garrigue, macchia and oakwoods display a vegetation progression typical for the Tyrrhenian Coast. But the formation of the terrain and its plant communities is determined by the maintenance of pathways through the underwood and manmade constructions as buildings, ruins of the antic age, the second world war and the modern times, as well as railways, sewers, fences and concrete walls. In north-south direction the railways of the Circumflegrea intersect the whole area, parallel guided by a partially covered sewer, as well as many footpaths and some constructed walking trails and unsurfaced roads. The area east of the railways/waterway mostly consists of stone oak woods and dense macchia. The wood area in vicinity of the Promontorio di Cuma has recently been supplemented with wooden pathways and resting sites. On the western side the macchia merges into garrigue and coastal vegetation. The oak wood extends 1 km in northern direction (not shown on the map) behind the urbanization/balnearios of Marina di Licola. To a high degree the area's floristical richness is the result of manmade intrusions. As the whole area is intersected by major railways, roads and paths from north to south and numerous of minor footpaths in east-west direction cut through the underwood, a complex net of migration ways between the plant communities has been built. In effect, the sum of all these derelict wallings, ruderal sites and routes through the underwood increase the space of species rich transitional areas between different habitats.
The orchid genus *Ophrys* is beneficiating from this artificial situation. A survey of the investigation area revealed high densities of *Ophrys* plants along and on

human routes, on construction waste dump sites, and close to concrete grounds. They grow in enriched loose sands in open and semi-open situations. Only a few individuals grow dispersed in mossy, open spaces between the depressed evergreen bushes of the garrigue. As *O. sphegodes s.l.* is bound to disturbed, semi-natural habitats, it can be assumed that the large populations sizes and maybe also its existence along the Campanian Coast is dependent on the type of land-use. This part of the Campanian Coast (Cuma) is colonized since the 8th century BC. by Greeks and later Romans. In this light it seems plausible, that the assumed pollinator switch from *A. nigroaenea* to *A. bimaculata* happened close to Cuma. The bee *A. bimaculata* lives on sandy grounds. Therefore it is also unlikely that *A. bimaculata* was adopted by the populations on Vesuvio, where it grows on magma rocks. But both habitats provide sour grounds, rare in *Ophrys*. Supported by low Φ_ST values a close relationship between the SPH-GAR and the SPH-VES populations is indicated. The SPH-VES population is very isolated. Drift might have caused a scent alteration as prerequisite for the colonisation of the coast, where *O. sphegodes s.l.* shares the disturbed sandy habitat with its pollinator *A. bimaculata*. In such way displaying a case of progenitor-derivative parapatric speciation, where the progenitor is not affected by the descendant population.

As nearly all *Ophrys* species grow on poor and open soils, the human colonisation of the Mediterranean Area and connected to that, deforestation and agricultural land-use has surely facilitated the expansion of *Ophrys* in general, and its diversification and occupation of new ecological niches in particular. We propose that environmental disturbance due to human activity is the driving force of ecological speciation in the genus *Ophrys*. Secondary contact of previously isolated lineages, and the fast radiation into the new semi-natural habitats can serve as an explanation for the observed lack of genetic differentiation (Devey 2008, own unpubl. data), the occurrence of gene-flow (Soliva & Widmer 2003), and the sharing of quite different allele types among closely related Ophrys species (own unpubl. data).

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