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Why are there so many bee-orchid species? Adaptive radiation by intra-specific competition for mnesic pollinators

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ABSTRACT

Adaptive radiations occur mostly in response to environmental variation through the evolution of key innovations that allow emerging species to occupy new ecological niches. Such biological innovations may play a major role in niche divergence when emerging species are engaged in reciprocal ecological interactions. To demonstrate coevolution is a difficult task; only a few studies have confirmed coevolution as driver of speciation and diversification. Herein we review current knowledge about bee orchid (*Ophrys* spp.) reproductive biology. We propose that the adaptive radiation of the Mediterranean orchid genus *Ophrys*, comprising several hundred species, is due to coevolutionary dynamics between these plants and their pollinators. We suggest that pollination by sexual swindling used by *Ophrys* orchids is the main driver of this coevolution. Flowers of each *Ophrys* species mimic a sexually receptive female of one particular insect species, mainly bees. Male bees are first attracted by pseudo-pheromones emitted by *Ophrys* flowers that are similar to the sexual pheromones of their females. Males then are lured by the flower shape, colour and hairiness, and attempt to copulate with the flower, which glues pollen onto their bodies. Pollen is later transferred to the stigma of another flower of the same *Ophrys* species during similar copulation attempts. In contrast to rewarding pollination strategies, *Ophrys* pollinators appear to be parasitized. Here we propose that this apparent parasitism is in fact a coevolutionary relationship between *Ophrys* and their pollinators. For plants, pollination by sexual swindling could ensure pollination efficiency and specificity, and gene flow among populations. For pollinators, pollination by sexual swindling could allow habitat matching and inbreeding avoidance. Pollinators might use the pseudo-pheromones emitted by *Ophrys* to locate suitable habitats from a distance within complex landscapes. In small populations, male pollinators would disperse once they have memorized the local diversity of sexual pseudo-pheromone bouquets or if all *Ophrys* flowers are fertilized and thus repel pollinators *via* production of

repulsive pheromones that mimic those produced by fertilized female bees. We propose the following evolutionary scenario: *Ophrys* radiation is driven by strong intra-specific competition among *Ophrys* individuals for the attraction of species-specific pollinators, which is a consequence of the high cognitive abilities of pollinators. Male bees record the pheromone signatures of kin or of previously courted partners to avoid further copulation attempts, thereby inducing strong selection on *Ophrys* variation in odour bouquets emitted by individual flowers. The resulting odour bouquets could by chance correspond to pseudo-pheromones of the females of another bee species, and thus attract a new pollinator. If such pollinator shifts occur simultaneously in several indivuals, pollen exchanges might occur and initiate speciation. To reinforce the attraction of the new pollinator and secure prezygotic isolation, the following step is directional selection on flower phenotypes (shape, colour and hairiness) towards a better match with the body of pollinator's female. Pollinator shift and the resulting prezygotic isolation is adaptive for new *Ophrys* species because they may benefit from competitor-free space for limited pollinators. We end our review by proritizing several critical research avenues.

Key words: adaptive radiation, sympatric speciation, pollination, sexual swindling, plant–insect coevolution, asymmetric coevolution, chemical ecology, *Ophrys*, orchid, unified species definition, pseudocopulation, key innovation.

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I. INTRODUCTION

Understanding how and why the diversity of life on earth increased over time is a key research question for biologists (e.g. Hutchinson, 1959; Wilson, 1992). In *On the Origin of Species*, Darwin (1859) proposed that species evolved according to an unbalanced speciation/extinction process, with more new species branching on the tree of life than are pruned by extinction. In adaptive radiations, this process is even more unbalanced and radiating groups experience a rapid diversification of species that colonize a variety of ecological niches (Schluter, 2000; Gavrilets & Losos, 2009). Recent syntheses proposed that radiations occur mainly through the evolution of key eco-morphological innovations that allow emerging species to occupy new ecological niches (e.g. Simões *et al*., 2016; Strouds & Losos, 2016). Most, if not all radiations studied thus far involve the evolution of such key innovations in response to environmental changes.

Rapid phenotypic evolution and hence the evolution of key novelties are likely to occur when a few species are engaged in narrow ecological interactions (e.g. Grant & Grant, 2006; Litsios *et al*., 2012). When their survival depends on the survival of their respective partner(s), species mutually force each other(s) to adapt, which entails coevolution (Solé & Sardanyés, 2014). Coevolution has long been regarded as one of the major processes organizing biodiversity (Ehrlich & Raven, 1964, but see Suchan & Alvarez, 2015), although it is still unclear when and how it generates species diversity (Thompson, 2016). To demonstrate coevolution in the real, natural world is a difficult task (Gomulkiewicz *et al*., 2007) and so far, evidence confirming that coevolution is a driver of speciation and

diversification remains elusive (Althoff, Segraves & Johnson, 2014; Hembry, Yoder & Goodman, 2014; but see Parchman, Benkmans & Britch, 2006).

This lack of direct evidence for speciation by coevolution contrasts with the pre-eminent role attributed to coevolution between plants and their biotic pollinators in the exceptional radiation of angiosperms (Bascompte & Jordano, 2007; Schatz *et al*., 2017). Plant–pollinator coevolution is presented as the solution to "Darwin's abominable mystery", i.e. the rapid rise and diversification of angiosperms (Davies *et al*., 2004). This discrepancy could be due to a more general problem in eco-evolutionary theory that is the prevailing, but often untested, idea that ecological and evolutionary processes act on different modes and at different tempos (Hairston *et al*., 2005; Schoener, 2011). Studies on closely related taxa indeed confirm that their reproductive isolation, and hence their speciation, may have occurred in only a few generations, for example under selection by extreme environmental changes (e.g. Lamichhaney *et al*., 2015, 2016; Parchman *et al*., 2016). The separation of processes acting on ecological timescales from those that occur on evolutionary timescales may thus be particularly misleading when considering the role of coevolutionary processes in speciation, by blurring the fact that adaptations gained by one or several partners involved in coevolution could induce reproductive isolation.

The explosive speciation rate of the genus *Ophrys* (bee orchids) is among the highest reported in angiosperms, with diversification rates peaking at between 4 and 8 lineages million yr^{-1} in some clades (Breitkopf *et al*., 2015). This spectacular radiation has given rise to several hundred species in the Mediterranean region of the western Palaearctic (Delforge, 2016) since the origin of this genus *ca*. 4.9 10^6 yr ago (Breitkopf *et al*., 2015). Here we propose that this spectacular adaptive radiation is due to the particular coevolutionary dynamics between these plants and their pollinators. We suggest that the unusual pollination mechanisms used by bee orchids are the main driver of this coevolution. Bee orchids use

pollination by sexual swindling: flowers of each *Ophrys* species mimic sexually receptive females of one particular species of insect, mainly solitary bees (Hymenoptera). Males of the corresponding species are lured by efficient sexual stimuli produced by the flowers (e.g. Schiestl *et al*., 1999). They are attracted by a blend of organic compounds emitted by *Ophrys* flowers that mimic the sexual pheromone odour bouquet of their females (pseudo-pheromones). The phenotypes of the flowers induce males to land and attempt to copulate with the flower, i.e. pseudo-copulation. Pollen is glued onto the male's body (generally his head or abdomen) as a result of his movements on the flower. Pollen is transferred to the stigmatic cavity of another flower of the same *Ophrys* species during a similar copulation attempt (e.g. Johnson & Schiestl, 2016).

Pollination by sexual swindling, the most specialized pollination strategy in orchids (Scopece *et al*., 2007), is employed by hundreds of species worldwide, and new examples continue to be discovered (Bohman *et al*., 2016). The preeminence of this strategy in orchids contrasts with the scarcity of its use in other angiosperms, with only two cases currently known (Bohman *et al*., 2016). Within the Orchidaceae, this strategy has evolved repeatedly with pollinators belonging to Hymenoptera, and possibly to Coleoptera and Diptera (Bohman *et al*., 2016). Besides *Ophrys*, the best-studied examples are from Australia and concern the pollination of species belonging to the genera *Chiloglottis* and *Drakaea*, which both attract Thyninne wasps as pollinators (Bohman *et al*., 2016).

Evolutionary specialization in plant–pollinator interactions refers to the process by which ecologically or phenotypically less-specialized species become more specialized (Armbruster, 2017). However, symmetric coevolution in which simultaneous evolution of flowers towards more specialized pollination, and of pollinators towards more specialized use of floral resources occurs is rare (Armbruster, 2017). Here we propose an asymmetric coevolutionary relationship between *Ophrys* and their pollinators, in which the plants rely

strictly on insects for their reproduction, whereas pollinator rewards involve male (re)location in suitable habitats with benefits for both male and female reproductive success and hence consequences on their population spatial dynamics.

Our scenario of asymmetric coevolutionary dynamics between *Ophrys* and their pollinators relies on three hypotheses that we include in the conceptual framework of adaptive radiation introduced by Bouchenak-Khelladi *et al*. (2015). This framework contrasts the extrinsic, environmental context with the biological, intrinsic traits, i.e. whether they provide background conditions, trigger the radiation, or modulate the radiation.

First, we assume that there is strong intra-specific competition among *Ophrys* individuals for attraction of species-specific pollinators, due to the high learning and memory (mnesic) abilities of these hymenopterans that record the pheromone signatures of kin or of previously courted partners. Pollinator limitation is widely observed in angiosperms (e.g. Van der Niet & Johnson, 2012; Van der Niet, Peakall & Johnson, 2014), but this is exacerbated by the refined cognitive processes of most *Ophrys* pollinators. Mnesic pollinators thus induce a strong selection pressure for variation in the pseudo-pheromone bouquets emitted by the flowers. Variation of odour bouquets among *Ophrys* individuals could by chance correspond to the pseudo-pheromones of another pollinator, and thus attract a new pollinator to the flowers. If such pollinator shift occurs simultaneously in spatially close *Ophrys* individuals (i.e. within the patrolling range of pollinators), pollen exchanges might occur and initiate a speciation process. Thus, mnesic pollinator limitation and the variability of the pseudo-pheromone bouquets emitted by the flowers would represent extrinsic and intrinsic conditions, respectively, triggering the radiation of *Ophrys* species according to the framework of Bouchenak-Khelladi *et al*. (2015). Pollinator shifts would be adaptive for the new *Ophrys* species because newly divergent individuals would experience relaxed pollinator limitation and thus would benefit from a competitor-free space. Such shifts could be facilitated by the

availability of a large number of potential pollinator species. Indeed, in contrast to most angiosperms, the diversification and radiation of *Ophrys* began after hymenopterans were already highly diversified (Condamine, Clapham & Kergoat, 2016). This availability of a wide pool of potential pollinators would have provided the background conditions Bouchenak-Khelladi *et al*., 2015) for the radiation of *Ophrys* species.

Secondly, we assume that the shift towards a new pollinator takes place due to the random attraction of a syntopic member of the pollinator guild with respect to that particular *Ophrys* population. This individual, random variation in pseudo-pheromone bouquets would then be followed by directional selection on flower phenotypes and on flowering period to reinforce attraction of the new pollinator and hence the reproductive success of the new *Ophrys* species. Directional selection is an intrinsic trait that would modulate the radiation of *Ophrys* species (Bouchenak-Khelladi *et al*., 2015).

Third, we assume that pollinators would use the pseudo-pheromone bouquets emitted by *Ophrys* to locate suitable habitats from a distance within complex landscapes. There is a perfect match between the habitats of *Ophrys* and of their pollinators (Paulus 2006, Vereecken et al. 2007, Paulus, 2017, 2018). Pollinators may remain in these habitats attracted by the local diversity of pseudo-pheromone bouquets, which may increase their probability of encountering a receptive female and hence the reproductive success of both sexes. Conversely, pollinators may disperse out of small suitable habitats once they have memorized all the local diversity of sexual pseudo-pheromone bouquets or if fertilized *Ophrys* flowers repel pollinators. Such dispersal will be advantageous to the plant by decreasing the probability of geitonogamy. It may also limit pollinator mating with locally emergent insect females, and hence limits inbreeding and favours gene flow (pollinator advantage). These feedbacks between pollinator spatial dynamics and plant population dynamics are at the heart of the coevolution between *Ophrys* and their pollinators: the two partners potentially could

benefit from their mutual association. However, the relation is asymmetrical because the plant is reliant on the pollinator whereas the reverse is not true.

The main goal of our approach is to provide a coherent explanation for rapid speciation in *Ophrys* driven by intra-specific competition for limited, mnesic pollinators. We first present briefly some general features of the biology of *Ophrys* species and their pollinators. We then screen the literature to investigate empirical support for the three hypotheses described above and hence for our scenario of asymmetric coevolution between *Ophrys* and their pollinators. We review the inter-specific interactions leading to pollination by sexual swindling, in the context of intra-specific competition between individual plants for mnesic and limited pollinators. We then discuss how this intra-specific competition could fuel speciation in sympatry and the rapid radiation in *Ophrys*. Finally, we prioritize the critical avenues for future research.

II. THE BEE ORCHIDS AND THEIR POLLINATORS

(1) Bee orchids

Ophrys is a monophyletic genus of orchids that are endemic to the western Palaearctic, mainly the Mediterranean region (Devillers & Devillers-Terschuren, 1994; Delforge, 2016). The Mediterranean region forms a band that covers *ca.* 4000 km in latitude, and is one of the largest archipelagos in the world with five main islands and about 10,000 islands and islets. This geographic situation combined with high geological diversity, a complex climatic history of glaciation–deglaciation cycles during the Quaternary with associated sea-level variation and land uplift and subsidence, regular fire disturbances and a signficant influence of humans has shaped a mosaic of landscapes with a wide variety of environmental niches (Blondel & Aronson, 1999). Most *Ophrys* species occur in mesic to dry grasslands, shrublands or light woodlands where the cover and height of the herbaceous layer are limited, and hence inter-

specific competition with other herbaceous species is rather low (Pridgeon *et al*., 2001; Eber, 2011; Jacquemyn & Hutchings, 2015). The spatial distribution of *Ophrys* populations maps onto the distribution of such habitats, and is generally discontinuous in the complex Mediterranean landscapes according to edaphic and mesoclimatic conditions, fire regimes and human land use.

Ophrys are allogamous (with the exception of *O. apifera* which is predominantly autogamous) long-lived plants (up to 20 years) that flower for 4–6 weeks once a year, although individual plants do not necessarily flower every year (Wells & Cox, 1991; Hutchings, 2010). *Ophrys* stems up to 15 flowers that open more or less successively (Delforge, 2016). Most *Ophrys* species [with two exceptions, the autogamous *O. apifera*, and *O. helenae* (Verrecken *et al*., 2012)] use sexual swindling to attract pollinators (Delforge, 2016). In this particular pollination syndrome, which was misunderstood by Darwin (1862), three different stimuli act successively in space and time (e.g. Fransisco & Ascensão, 2013). The flowers emit an odour bouquet of volatile organic compounds (pseudo-pheromones) that are similar to the sexual pheromones produced by virgin females of the pollinator species that attract males at a distance of up to 5–10 m (Paulus, 2006). The shape and the colour of the flower then induce the male pollinator to land on the flower (e.g. Rakosy *et al*., 2012, 2017; Paulus, 2018). The shape (Rakozy *et al*., 2017) and the hairiness (Ågren, Kullenberg & Sensenbaugh, 1984) of the flower orient the body of the male parallel to the longest length of the labellum on the flower. A pollinator landing on the labellum may detect by antennal contact pseudo-pheromones emitted by the flower that mimic the odour of a potential sexual partner, which leads the pollinator to initiate copulation behaviour (e.g. Mant *et al*., 2005*a*). The male extends his genital apparatus and probes the labellum with his abdomen; actual sperm deposition has been reported only by Willmer (2011). The pollinator is guided by the orientation of the flower either to the top of the labellum or to its base. This positioning

mechanism means that the coherent masses of pollen grains (pollinia) are glued to the part of the insect's body that is close to the anthers, this is either the head or abdomen in different orchid species (e.g. Kullenberg, 1961; Kullenberg & Bergström, 1976; Devillers & Devillers-Terschuren, 2013).

In *Ophrys*, pollen grains issued from a single mother cellare grouped into *massulae*. Massulae themselves are grouped into pollinia, with two pollinia present per *Ophrys* flower (e.g. Claessens & Kleynen, 2011, 2016). Copulation attempts end typically after 10–30 s, and the male leaves the flower (e.g. Claessens & Kleynen, 2011, 2016). He may be attracted to another conspecific flower where this behavioural sequence is repeated. This new copulation attempt can lead to the depositing of either massulae or the entire pollinia into the stigmatic cavity of the flower (e.g. Paulus, 2006). After its capture by a pollinator the pollinia bends forwards, favouring physical contact with the stigmatic cavity of the next flower (Johnson & Nilsson, 1999; Claessens & Kleynen, 2011, 2016).

Each fertilized flower can produce thousands of tiny seeds (e.g. Nazarov & Gerlach, 1997; Paulus, 2006) that are wind dispersed (e.g. Salisbury, 1975). These minute seeds have no nutrient reserves (Arditti & Ghani, 2000) and require interactions with mycorrhizal fungi to germinate. The presence of suitable species of fungi is key for *Ophrys* establishment, as in other orchids (e.g. McCormick & Jacquemyn, 2014; Jacquemyn *et al*., 2015; Rasmussen *et al*., 2015). Some phylogenetically close species are known to share mycorrhizal partners in sympatry, suggesting that mycorrhizal symbiosis has not played a role in reproductive isolation (Gervasi *et al*., 2017), as in other orchid genera (Schatz *et al*., 2010). The generation time from a seed to a reproductive plant is still not known precisely, but according to observations of seedling development, may take between two and several years (Fabre, 1852; Hutchings, 2010; Jacquemyn & Hutchings, 2015).

Species richness described within *Ophrys* varies from 9 to 354 species [summarized in Tyteca & Baguette (2017), Bateman (2018) and Bateman *et al*. (2018*a*)] according to the different definitions of 'species' used by different systematic authorities. The most conservative number results from the combination of three criteria for delineating a species (Bateman *et al*., 2011: similarity of appearance of individuals, reproductive isolation and monophyly. The critical point of this approach is its self-declared use of molecular markers to assess these three criteria. Molecular markers are indeed useful to infer individual relatedness or gene flow within and among metapopulations. However, use of monophyly among genomes to infer speciation is not in agreement with a genic view of speciation. According to Wu (2001) and Wu & Ting (2004), changes to a few genes under strong selection could lead to the evolution of isolation mechanisms without major genomic changes, which is not in contradiction with the biological definition of a species (Mayr, 2001).

Heated debate around the appropriate species definition to use within *Ophrys* (e.g. Bateman *et al*., 2011; Vereecken *et al*., 2011; Johnson & Schiestl, 2016; Bateman, 2018*a*) has led to suggestions that there is taxonomic exaggeration in European orchids ("orchid fever") due to the pre-eminence of splitters over lumpers among European taxonomists (Pillon & Chase, 2007). The repeated refusal of the lumpers to consider that prezygotic isolation, and hence speciation, occurred *via* pollinator shifts has led to the introduction of terms like 'macrospecies', 'microspecies' or ' prospecies' (Bateman *et al*., 2011, 2018; Bateman, 2018*a*). Although we acknowledge that some taxonomic exaggeration might exist, we believe that there is increasing evidence confidently to support reproductive isolation, evolutionary divergence and hence species formation in several hundred *Ophrys* taxa.

We thus use the unified species definition coined by de Queiroz (2005, 2007) that considers species as separately evolving metapopulation lineages. This definition was initially applied to the genus *Ophrys* by Devillers & Devillers-Terschuren (2013), who focused on the

evolution of phenotypic characters that are targets of selection eventually leading to speciation, and hence that are diagnostic of species delimitation. Herein, we show that this unified species definition is also useful to understand the emergence, establishment and maintenance of characters leading to the differentiation of metapopulation lineages, and hence to speciation, in *Ophrys*. Metapopulations are groups of local populations that are connected by dispersal (e.g. Hanksi, 1999). Within a landscape, individuals of a species restricted to certain areas form local populations where there are suitable conditions for them to complete their life cycle. This includes, for *Ophrys*, both aboveground (light, wind and pollinators) and belowground (pH, nutrients and fungi) elements. The landscape may be viewed as a mosaic of suitable habitat patches with or without local populations embedded within an area of unsuitable habitat, or matrix. If male gametes (pollen) or zygotes (seeds) leave their current habitat they have to disperse across the matrix and are therefore exposed to risks that may result in death. However, the dispersing male gametes or zygotes may reach another habitat patch with suitable above- and belowground conditions (or for gametes, with receptive flowering individuals). The overall result is that local populations are not isolated but are in fact connected by dispersal to form a metapopulation.

Metapopulations have properties that local populations do not possess. Firstly, the persistence of metapopulations is more stable than that of local populations because local extinctions can be counter-balanced by the creation of new populations elsewhere in the landscape following successful dispersal events (colonization). Secondly, the dynamic nature of a metapopulation (that is, gene flow associated with dispersal and local population turnover) contributes to its genetic structure and diversity, and hence to its evolutionary trajectory (e.g. Baguette, Michniewicz & Stevens, 2017). In *Ophrys* metapopulations, wind dispersal of seeds allows (re)colonization, while gene flow among local populations occurs through both seed dispersal and the dispersal of pollinia carried by pollinators among habitat

patches within landscapes. The complexity and timescale of these two types of dispersal are dramatically different, however: several years may elapse between the landing of a seed and its first flowering, whereas gene flow associated with pollinia transfer occurs on a yearly basis. Several studies reveal unambiguously that the reproductive success of individual *Ophrys* plants is low to very low (e.g. Claessens & Kleynen, 2011), although variable in space and time (Table 1). Three studies report that this crucial fitness parameter depends significantly on *Ophrys* population spatial structure (Vandewoestijne *et al*., 2009; Gervasi *et al*., 2017; Borràs & Cursach, 2018). Considering *Ophrys* species as separately evolving metapopulation lineages has the immense merit of putting the evolutionary dynamics associated with pollination by sexual swindling into a spatio-temporal context.

According to the unified species concept, prezygotic isolation associated with attraction of a specific pollinator species is a sufficient criterion to delineate an *Ophrys* species (Paulus, 2006, 2018; Paulus & Gack, 1990; Vereecken *et al*., 2011; Breitkopf *et al*., 2015). Leading *Ophrys* systematists concur with this view by stressing that, within this genus, the complex of adaptations leading to attraction of a specific pollinator within populations of the same lineage is the norm used to delimit a species (Devillers & Devillers-Terschuren, 2013). The integrative taxonomy approach (Dayrat, 2005; Padial *et al*., 2010; Johnson & Schiestl, 2016) integrates a wider array of biological characters than pollinator identity (e.g. plant and flower morphology and phenology, population genetic structure, ecological preferences). This approach usually confirms the species diagnosis determined by the attraction of a specific pollinator species, hence validating the use of the unified species definition (Joffard, Buatois & Schatz, 2016; Joffard *et al*., 2020).

On this basis, we consider that the genus *Ophrys* includes several hundred species in the Western Palaearctic (Delforge, 2016), which can be separated by the convergence of pollinator identities and plant phenotypic characters, rather than 9–11 species separated by

genome dissimilarity (Bateman, 2018; Bateman *et al*., 2018*a*), which rather correspond to 9–11 different clades. The use of DNA sequences for *Ophrys* systematics is currently a conundrum; the most comprehensive study is a time-calibrated phylogeny based on the analysis of the sequences of six nuclear loci in 37 *Ophrys* species by Breitkopf *et al*. (2015), which showed that the diversification rate of *Ophrys* species was not constant over time. Overall, this phylogeny is consistent with the results of Bateman *et al*. (2018*a*), based on 34 accessions and a genotyping by sequencing (GBS) protocol that resulted in a set of 4159 single nucleotide polymorphisms (SNPs), although these phylogenies differ in their placement of the *Ophrys insectifera* clade. Since the putative origin of the group (*ca*. 4.9 million years ago), the radiation of the *Ophrys* genus seems to involve an increase in diversification rates of particular clades, which can be related to possible pollinator shifts within these clades (Breitkopf *et al*., 2015; Fig. 1). However, how and why such shifts occurred remain open questions.

(2) Bee orchid pollinators

Most currently known pollinators of *Ophrys* (>98%) are males of Aculeata (Hymenoptera) species belonging to several families of solitary bees (Apidae, Megachilidae, Colletidae, Halictidae and Andrenidae). Exceptions are males of one species of sawfly (Hymenoptera: Symphita: Argidae), two species of Crabronidae (Hymenoptera: Aculeata), one species of colonial bees (*Bombu*s; Hymenoptera: Apidae), two species of syrphid flies (Diptera: Syrphidae), two species of scarabeid beetles (Coleoptera: Scarabeidae) and one species of beetle (Coleoptera: Oedemeridae) (Classens & Kleynen, 2011, 2016; Gaskett, 2011; Delforge, 2016; Paulus, 2018; Joffard *et al*., 2019; Schatz *et al*. 2020).

The taxonomy of the *ca.* 2000 wild bee species in Europe was recently reviewed (e.g. Danforth *et al*., 2013; Fortel *et al*., 2014). Solitary bees are particularly diverse and abundant

within the Mediterranean region, in habitats similar to those described above as suitable for *Ophrys* species (Rasmont & Haubruge, 2014). In solitary bees, the adult stage usually lasts for only three or four weeks, and some species have two or even three adult generations per year. During their brief adult life, males will patrol nesting areas or *rendez-vous* sites hoping to mate with a female (Paxton, 2005). Females of most species will mate only once soon after emergence from pupae – they store the sperm and release it when needed – and then spend their time creating and provisioning a nest in which to lay their eggs. Female solitary bees have impressive engineering skills, and go to extraordinary lengths to construct a secure nest, as carefully documented by skilled natural historians (Ferton, 1923; Fabre, 1924). Most species nest in the ground, digging a tunnel into bare or partially vegetated, well-drained soil or use abandoned beetle burrows or other tunnels.

The mode of sex determination in these hymenopterans is arrhenotokous parthenogenesis (arrhenotoky), in which fertilized eggs develop as females and unfertilized eggs develop as males (Heimpel & De Boer, 2008). Arrhenotoky is the dominant and ancestral pattern of sex determination in Hymenoptera, and the only pattern found in the superfamilies to which *Ophrys* pollinators belong (Heimpel & De Boer, 2008). Arrhenotoky is a subset of haplodiploidy, a genetic system in which females are diploid and males are haploid (Heimpel & De Boer 2008). As males emerge from unfertilized eggs, arrhenotokous parthenogenesis prevents transfer of genetic material from a father to its male offspring. This particularity prevents hymenopteran males from easily evolving counter-adaptations to the *Ophrys* flower pollination strategy consisting in closely mimicking the various signals corresponding to a conspecific female.

Arrhenotokous parthenogenesis associated with single-locus complementary sex determination renders hymenopterans highly sensitive to inbreeding. In addition to its various deleterious effects (e.g. Charlesworth & Charlesworth, 1987), inbreeding can lead to the

production of diploid, infertile males in hymenopterans (e.g. Zayed & Packer, 2005). These insects have thus developed three mechanisms of inbreeding avoidance: (1) kin recognition, i.e. the recognition and avoidance of kin as potential mates (e.g. Pusey & Wolf, 1996); (2) protandry, i.e. adult males emerge from their pupae and are active one or two weeks before females, (e.g. Eickwort & Ginsberg, 1980); and (3) dispersal, with one sex, usually males, more likely to disperse and dispersing further than the other (Gandon, 1999; Perrin & Mazalov, 1999). Maximum dispersal distances recorded in male eusocial bees were around 2.5 km (dos Santos, Imperatriz-Fonseca & Arias, 2016), with most having a foraging range of several hundreds of meters (Gathman & Tscharntke, 2002; Zurbuchen *et al*., 2010).

Behavioural observations of Hymenopterans showed that mating with kin is avoided by the emission by both sexes of an individual odour signature (Ayasse, Paxton & Tengö, 2001). Laboratory and field studies of solitary bees show that females produce individually distinctive sexual pheromones that are attractive to males (Wcislo, 1987, 1992). There is considerable inter-individual variation in female attractiveness to males among sexually immature females (Wcislo, 1987, 1992), and males from the same population show striking consistency regarding female attractiveness (Wcislo, 1987). Males use sexual pheromone signals to learn the identity of female bees with which they have attempted to copulate, thereby avoiding these females in future encounters (Smith & Ayasse, 1987; Wcislo, 1987, 1992). Female pheromone signals also inform about their receptivity, thus acting as female olfactory 'identity cards' that allow males to save time by not attempting to copulate with non-receptive females (Barrows; Bell & Michener *et al*., 1975; Wcislo, 1987, 1992).

The chemical basis of individual identity recognition in hymenopterans has been investigated for the solitary bee *Colletes cunicularius* (Vereecken, Mant & Schiestl, 2007). Using electro-antennography, Vereecken *et al*. (2007) identified physiologically active compounds in solvent extracts of virgin females. Electro-antennography detects changes in

electric potential from an insect antenna exposed to an organic compound that result from the superimposition of simultaneous membrane depolarisations of numerous receptor cells; thus they are indicative of an output from the antenna to the brain (reviewed by Schiestl & Marion-Poll, 2002). Via this output, the organic compound can trigger receptor potentials in olfactory neurons (Schiestl & Marion-Poll, 2002). Vereecken *et al*. (2007) detected individual variability in physiologically active compounds that was higher among than within populations of *Colletes cunicularius*, implying the existence of population-specific 'dialects' within a multidimensional 'olfactory landscape'. Comparisons of the preferences of males of known provenance for synthetic copies of female sex pheromones showed that they were attracted significantly more often by odour types from allopatric populations, perhaps as a mechanism to prevent inbreeding (Vereecken *et al*., 2007).

III. ORCHID–POLLINATOR INTERACTIONS

The three mechanisms (i.e. kin and mate recognition, protandry and dispersal) by which hymenopterans avoid inbreeding or the loss of time and energy in mating with previously mated partners each play a role in the coevolutionary relationship between *Ophrys* and their pollinators, and hence may have potential roles in the speciation and adaptive radiation of these orchids. *Ophrys* flowers simulate and manipulate insect kin- and mate-recognition mechanisms to attract their pollinators. *Ophrys* flowering is synchronous with the emergence of their pollinators from their pupae, earlier than virgin females. Pollinated *Ophrys* flowers emit anti-aphrodisiac compounds similar to pheromones released by mated hymenopteran females to avoid further courtships that repel pollinator males, as documented for *Ophrys sphegodes–Andrena nigroaenea* (Schiestl & Ayasse, 2001). We now review the empirical evidence associated with each of these three mechanisms, to clarify how intra-specific

competition between plants could drive the speciation mechanisms that underlie the adaptive radiation in *Ophrys*.

(1) Pollinator attraction by manipulation of kin- and mate-recognition mechanisms

(*a*) *Pseudo-pheromones mimic attractive insect female sexual pheromones*

The potential role of flower odours emitted by *Ophrys* in the attraction of pollinators was first suggested by Correvon & Pouyanne (1916, 1923) and Pouyanne (1917). Borg-Karlson (1990), based on a hypothesis developed by Kullenberg (1961), used electrophysiological tests [gas chromatography coupled with electro-antennographic detection (GC-EAD) and mass spectrometry (GC-MS)] to identify organic compounds emitted by *Ophrys* flowers or by females of their pollinators. In a sample of 30 *Ophrys* species, she identified compounds that triggered pollinator attraction but not those that were releasing factors of copulation behaviour. These results were confirmed by behavioural tests assessing the success of candidate organic compounds spread on insect dummies in attracting free-flying pollinators. Kullenberg (1961) thus proposed that *Ophrys* flowers produce only 'second-class attractive compounds' that are neglected if the pollinator females are present.

However, further experiments demonstrated that organic compounds emitted by *Ophrys* flowers could indeed stimulate pollinator copulation behaviour (Schiestl *et al*., 1999, 2000). Similarity between the organic compounds emitted by *Ophrys sphegodes* flowers and the sexual pheromones present in cuticular extracts from females of their pollinators (males of the solitary bee *Andrena nigroeanea*) was assessed using GC-EAD and GC-MS. There was extensive similarity in biological activity between female sexual pheromones and the *Ophrys* flower odour bouquet, including comparable composition and relative proportions of biologically active organic compounds. Synthetic, analogous blends of these compounds (C21–C29 n-alkanes and n-alkenes) applied to female dummies triggered copulation attempts

by male pollinators (Schiestl *et al*., 1999, 2000). For all *Ophrys* species investigated to date, copulation attempts of pollinators can only be elicited by a blend of organic compounds that is similar in composition and quantity to the female sexual pheromones of the pollinator (Schiestl *et al*., 1999, 2000; Ayasse *et al*., 2000, 2003; Schiestl & Ayasse, 2002; Mant, Peakall & Schiestl, 2005*b*; Ayasse, 2006; Stökl *et al*., 2005, 2007, 2008, 2009; Gögler *et al*., 2009, 2011; Vereecken & Schiestl, 2008; Cuervo *et al*., 2017; Gervasi *et al*., 2017).

The composition of the blend of organic compounds forming the floral analogues of sex pheromones seems to vary according to the systematic position of their hymenopteran pollinators (Cuervo *et al*., 2017). Wasp-pollinated *Ophrys* species attract their pollinators by using polar hydroxyacids, *Eucera*-pollinated *Ophrys* species use a mixture of polar and non-polar compounds and *Andrena*-pollinated *Ophrys* species use non-polar hydrocarbons (Cuervo *et al*., 2017). This variation may correspond to pollinator shifts during the evolutionary history of the genus *Ophrys* (Breitkopf *et al*., 2015). Pollinator shifts are frequent sources of speciation in angiopserms, being involved in *ca*. 25% of divergence events (van der Niet & Johnson, 2014). However, shift frequency and directionality vary extensively, in response to variation in intrinsic factors such as floral features and phylogenetic history, as well as extrinsic factors such as interactions with local pollinator assemblages (van der Niet & Johnson, 2012). In *Ophrys*, pollinator shifts could have promoted the rapid diversification observed within several clades (Fig. 1; Breitkopf *et al*., 2015), although this has yet to be demonstrated. Firstly, clades in which rapid diversification occurred are not pollinated by a single pollinator family (Table 2) (Gaskett, 2011; Breitkopf *et al*., 2013; Joffard *et al*., 2020). Secondly, the analyses of the composition of pheromone analogues within the *Ophrys insectifera* clade, which is the most basal clade according to most molecular phylogenetic hypotheses [Fig. 1; but see Bateman *et al*. (2018*a*) and Piñeiro-Fernandes *et al*. (2019)] using DNA sequence data, showed the simultaneous emission of both

esters and non-polar hydrocarbons (Joffard *et al*., 2016, Gervasi *et al*., 2017). Moreover, among the three species in this clade, *O. aymoninii*, which is the only one to be pollinated by a species of *Andrena* (*A. combinata*), is also the only one to emit esters (Joffard *et al*., 2016). Unfortunately, we still lack data on the attractiveness of these compounds to the wasps that pollinate *O. insectifera*.

The main message we can draw from these studies is the striking similarity in the composition and relative amounts of the constituents of the scents emitted by orchid labella and the sex pheromones of female bees. These similarities support the view that a specific blend of organic compounds was a crucial key innovation of *Ophrys* to attract pollinators, and, importantly, to swindle male pollinators into the initiation of copulation behaviour.

(*b*) *Intra-specific variation in organic compounds in* Ophrys

Ayasse *et al*. (2000) identified 106 compounds in the odour bouquets of *Ophrys sphegodes* flower extracts. GC-EAD revealed that only 24 of these triggered stimulation of male pollinator antennae. Their data indicated unambiguous variation among inflorescences of the same species in GC-EAD active compounds. Moreover, flowers of the same inflorescence showed a higher similarity in odour bouquet than flowers of different inflorescences, but differed in their relative proportions of GC-EAD-active aldehydes and esters. The authors proposed that such variability is important to avoid pollinator habituation, thus favouring subsequent visits to flowers on the same inflorescence by a given pollinator. During behavioural experiments, two-thirds of males that visited one flower also visited a second flower on the same inflorescence. Mixtures of GC-EAD-active compounds from inflorescences separated by 30 km were significantly more different among populations than within populations, suggesting variation of olfactory signals among metapopulations. These differences were due to variation in the relative proportions of n-alkanes and n-alkenes within

the blend of GC-EAD-active organic compounds. Finally, GC-EAD- active compounds showed less intra-specific variation in odour bouquets compared to non-active compounds, suggesting that variation of pollinator-attracting communication signals is constrained in some way.

Mant *et al*. (2005*b*) investigated the chemical compounds present in the flower labella of *Ophrys exaltata* using a split geographical design: individual variation was assessed among regions, among populations within regions and within populations. The authors identified 59 compounds, of which 22 were identified as behaviourally active in the pollinator *Colletes cunicularius* by inducing male landing and copulation attempts on compound-impregnated dummies. Variance partitioning indicated that one-third of the odour variation was due to individual differences in physiologically active compounds within conspecific populations. Moreover, most of the individual variation (60%) was observed among regions, i.e. among metapopulations, whereas only a small fraction of the variance (7%) was observed among local populations. Strikingly, as in the previous study (Ayasse *et al*., 2000), inactive compounds present in the flower labella were much more different amongst individuals: variance partitioning indicated that 95% of the observed variation was within populations for behaviourally inactive compounds.

Vereecken & Schiestl (2008) sampled plants and pollinators in 15 populations (13 allopatric and two sympatric) of the same plant–pollinator couple, *Ophrys exaltata–Colletes cunicularius*. They focused on odour bouquets involving three key organic compounds detected by male bees. They showed that odour compounds triggering stimulation of the male bee antennae (physiologically active compounds), differed markedly between orchid flowers and female bees, irrespective of their geographic origin. These differences in odour bouquets were even consistent in the two sympatric populations. Behavioural tests indicated that pollinators showed a marked preference for the odours of females from allopatric populations,

i.e. among metapopulations, confirming the previous results of Vereecken *et al*. (2007). Finally, males were always more attracted in behavioural tests by *O. exaltata* flowers than by female bees. The absolute amounts of biologically active compounds produced by the flowers and female bees were similar. The higher attractiveness of the orchid blends was thus suggested to be due to the changes in the relative amounts of the three organic compounds used. This result was confirmed by manipulating the ratios of the three key compounds in natural sex pheromone extracts of local females of *Colletes cunicularius*. Adding synthetic hydrocarbons to change the proportions present in the female bees' natural pheromones to match the mean ratios found in orchid floral odour samples led to a significantly higher attraction of patrolling male bees.

We can conclude from these experiments that within a given *Ophrys* species, each individual plant has its own olfactory signature from a pollinator viewpoint, which is produced by varying the relative amounts of physiologically active organic compounds produced by the flowers. In *Ophrys sphegodes*, although flowers of the same plant have a more similar olfactory signature than flowers of different plants, they still differ from each other in the relative amounts of some if their organic consistuents. These three experiments also indicate significant differences in the odour signatures among individuals belonging to different metapopulations. Further research should investigate: (1) the generality of the similarity between flowers of the same plant relative to flowers from other plants, which has so far been demonstrated only for one species; and (2) the potential roles of the GC-EAD-inactive organic compounds that seem much more variable than the active organic compounds. Note that a compound that is GC-EAD-active in one pollinator species may not be GC-EAD-active in another pollinator species, thereby adding to the hidden complexity of the system. We suggest that two non-exclusive explanations might generate the observed patterns: (1) pollinator-mediated selection to maintain the relative ratios of physiologically

active organic compounds (Raguso, 2008); and (2) a bet-hedging strategy (e.g. Beaumont *et al*., 2009) leading to high variability of physiologically inactive compounds allowing the production of new molecules that are potential pollinator attractors.

(*c*) *Intra-specific competition drives random crossing of peaks in the olfactory landscape*

Highly species-specific mutualistic or antagonistic interactions between plants and pollinators have been suggested to be mediated by a few, system-specific compounds through 'private channels' (Raguso, 2008; Chen *et al*., 2009). In contrast to highly species-specific mutualistic interactions, such as in the fig–fig wasp system, in which mutual benefits depend on the consistency of plant olfactory signals over time (Chen *et al*., 2009; Hossaert-McKey *et al*., 2010), olfactory signalling in the *Ophrys*–pollinator system cannot be stabilized over time due to the constraints imposed by mnesic pollinators, which counter-select the evolution of private channels (Dormont, Joffard & Schatz, 2019).

Two different processes have been invoked to explain the high level of within-species inter-individual heterogeneity in organic compounds emitted by *Ophrys* flowers. Vereecken & Schiestl (2008) proposed a proximal explanation based on pollinator-male preferences for novel signals. They suggested that pollinator populations are "probably subjected to inbreeding", which makes preferences for novel signals adaptive because it promotes outbreeding, i.e. it avoids sibling mating. They noted that such preferences for novel signals are a common feature in animal cognitive processes, and that this phenomenon can be an important driving force behind signal evolution (e.g. Lynn, Cnaani & Papaj, 2005; ten Cate & Rowe, 2007; Dormont, Joffard & Schatz, 2019). On the other hand, Schiestl (2005) proposed an ultimate explanation based on negative frequency-dependent selection: the high variability in floral signals may be maintained by negative frequency-dependent selection, since pollinators can learn and subsequently avoid common deceptive morphs more quickly than

rare ones. Ayasse, Gögler & Stökl (2010) considered the evolution of floral variation in *Ophrys* to be an extreme form of negative frequency-dependent selection in which there is counter-selection against similarity.

The first, proximal explanation is consistent with our current understanding of pollinator biology (see Section II.2), i.e. the high risk of inbreeding associated with the haplodiploidy mechanism of sex determination in hymenopterans, and the evolution of individual chemical signatures to facilitate kin and receptive-mate recognition (Barrows, Bell & Michener *et al*., 1975; Smith & Ayasse, 1987; Wcislo, 1992; Ayasse *et al*., 2001). We find less support for the second, ultimate explanation. In negative frequency-dependent selection the fitness of a discrete phenotype increases as its frequency in the population decreases, leading to balanced polymorphism, i.e. the long-term coexistence of several discrete phenotypes (i.e. morphs) that are selected according to their frequencies within the population [Brisson (2018) and references therein]. Such negative frequency-dependent selection is key in polymorphic plants that use the food-deception pollination syndrome. In such species, the preference of pollinators for a rarer phenotype progressively increases the relative frequency of this morph over generations, progressively decreasing its attractiveness to the benefit of the formerly frequent phenotype that beomes more attractive as it becomes rarer (e.g. Gigord, MacNair & Smithson, 2001; but see Jersáková *et al*., 2006). High inter-individual differences in *Ophrys* flower odour profiles do not correspond to this pattern of coexistence of discrete phenotypes. Moreover, many *Ophrys* populations may not be of sufficient size for negative frequency-dependent selection to have an effect.

We suggest that the variability in biologically active and inactive organic compounds emitted by *Ophrys* flowers is the result of intra-specific competition for a limited resource: their mnesic pollinators (see Hypothesis 1 in Section I). Intense competition for pollinators might promote individual variation in flower phenotypes of organic compound emission, and

this increased diversity might arise with plasticity alone, without the genetic changes that are theoretically assumed to be present (e.g. Svanbäck & Bolnick, 2007). Whatever the relative roles of genetic or epigenetic mechanisms, we suggest that the end result of high individual variability in organic compound emission could allow chance similarity with pheromones of females of another bee species, and thus attract a new pollinator. To use the metaphor of adaptive landscapes coined by Wright (1986), we propose that the chance emission of a particular blend of odours that attracts a new species of pollinator corresponds to the random crossing of peaks in the olfactory landscape (Vereecken *et al*., 2007; Svensson, Strandh & Löfstedt, 2014). If such pollinator shift occurs simultaneously in several individuals, pollen exchanges might occur and initiate speciation. Finally, we predict that if high competition for mnesic pollinators generates selection for random inter-individual variability in organic compounds, there should be a skewed reproductive success in *Ophrys* populations, with some individual plants matching the odour preferences of their pollinators better than others. Indirect evidence implies that this is indeed the case, with huge accumulations of pollinia within some flowers (Fig. 2).

(*d*) *Evidence for intra-specific competition and pollinator limitation*

There are three indirect indications of pollinator limitation in *Ophrys* that might induce intra-specific competition among conspecific individuals. Firstly, three studies analysing *Ophrys* individual fitness in a spatially explicit context found a negative relationship between reproductive success and conspecific density (Vandewoestijne et al. 2009, Gervasi et al. 2017, Borràs & Cursach, 2018). This general pattern is in good agreement with higher competition for pollinators in denser plant populations, and hence with pollinator limitation. Vandewoestijne *et al*. (2009) investigated the reproductive success of all plant individuals in four populations of *Ophrys sphegodes*, four populations of *Ophrys fuciflora* and five

populations of *Ophrys insectifera* (one with three sub-populations) over two successive years. Female individual reproductive success was approximated as fruit set (ratio of pollinated flowers or inflated capsules divided by the total number of flowers in a given inflorescence). For all three species examined, female individual reproductive success increased with increasing nearest-neighbour distance (i.e. the shortest distance to the nearest flowering individual). Moreover, female individual reproductive success generally increased with decreasing population density (number of individual plants/m²) and increasing habitat patch elongation (width/length). The latter parameter was included because a larger number of pollinators potentially are likely to encounter patch boundaries (and consequently immigrate into or emigrate out of the patch) when the perimeter increases. Fruit-set success was also variable between years, but in a similar way among populations and across species. Gervasi *et al*. (2017) investigated female individual reproductive success of 300 plants of the phylogenetically closely related *Ophrys insectifera* and *Ophrys aymoninii* that were randomly selected within six populations in which the two species lived in sympatry. They showed that in both species the pollination success of a given plant was negatively related to the number of conspecific individuals within a 2 m radius. Borràs & Cursach (2018) compared reproductive success among seven populations of *Ophrys balearica* during two successive years. They found that fruit set was higher in 2 × 2 m quadrats in which plant density was lower (1–10 individuals) compared to quadrats with higher density. Moreover, flowers that were located at the periphery of the populations had higher male reproductive success (measured by pollinia removal) than those at the centre of the populations. This negative relationship between pollination success and population size or plant density seems generalizable to rewardless plants due to competition for pollinator attraction (Johnson & Schiestl, 2016).

Secondly, many publications report that *Ophrys* flowers have a low to very low female individual reproductive success (e.g. Neiland & Wilcock, 1998; Claessens & Kleynen, 2011, 2016; Table 1). This general pattern is reflected by the rarity of a pollination event for an *Ophrys* individual, which again implies pollinator limitation. However, most publications do not discuss the extent of sampling used to compute this basic component of fitness. Vandewoestijne *et al*. (2009) and Borràs & Cursach (2018) showed that among the many parameters influencing the pollination of one or several flower(s) of a plant, both position relative to habitat borders and closest-neighbour distance influenced female individual reproductive success. Similar studies detailing exhaustively within-population success in relation to explicit spatial variables are clearly needed. Comparisons between male and female reproductive success estimated *via* the pollen transfer efficiency index would be a good way to evaluate intra-specific competition and its consistency across species and environmental contexts (Scopece, Schiestl & Cozzolino, 2015).

Thirdly, the life history of *Ophrys* individuals is rather unusual. They are long-lived (up to 20 years) herbaceous species (Wells & Cox, 1991; Hutchings, 2010) that produce large numbers of seeds (5000–20,000/fruit; Arditti & Ghani, 2000; Paulus, 2006; Claessens & Kleynen, 2011, 2016; Sonkoly *et al*., 2016) of tiny size (300–700 µm length, 100–200 µm width: Galán Cela *et al*., 2014) and mass ($11–20.10^{-7}$g; Sonkoly *et al*., 2016). At the time of dispersal, *Ophrys* seeds consist of a spindle-shaped, very slim seed coat that encloses an extremely small and simplified embryo formed of a spherical cluster of cells, which is a clear adaptation to wind dispersal (Arditti & Ghani, 2000). The embryo is unable to germinate on its own and has to engage in a mycorrhizal relationship with a fungus that sustains the development of the emerging seedling. Female individual reproductive success is thus dependent on the production of huge numbers of these wind-dispersed seeds that are capable of long-distance dispersal. Willems (1994) reported finding a single flowering *Ophrys apifera*

in a Dutch dune area that was 60–75 km distant from existing populations of this species. Theory predicts that selection for long-distance dispersal of seeds mediates positive density-dependent pollinator limitation in plant populations with deceptive pollination (Ferdy *et al*., 1999).

We are still lacking observational and experimental evidence of the link between individual variation in odour bouquet and reproductive success in a spatially explicit context, i.e. by considering *Ophrys* densities, habitat geometry and nearest-neighbour distance within local populations, and the intensity of gene flow within metapopulations (see Sletvold, Grindeland & Ågren, *et al*., 2010; Sletvold *et al*., 2016). Such research should also investigate the additive or interactive effects of potential explanatory variables influencing pollination success, such as pollinator abundances and weather conditions.

(*e*) *Evolution of floral odour phenotypes and speciation*

Strong intra-specific competition due to pollinator limitation would thus seem to favour the high inter-individual variability in organic compounds emitted by *Ophrys* flowers. We assume that there is selection on the flowers for the production of random blends of organic molecules that is mediated by their mnesic pollinators. The consequences of this selective pressure for novelty in odour bouquets produced by the flower would be threefold: (1) the resulting bouquet could be biologically inactive; (2) the bouquet could match the pheromones of virgin females of the usual pollinator species; and (3) the bouquet could match the pheromones of virgin females of another pollinator species. In the first case, there would be no chance of reproduction, and flower fitness would be nil. In the second case, cross-pollination would be possible if a flower–pollinator encounter occurs, and the match between a particular flower odour bouquet and the sexual pheromones of its pollinator will therefore maintain prezygotic isolation of this *Ophrys* species. This might explain the much higher

variability of biologically inactive compounds relative to active ones discussed in Section III.1*b*. The nature and the proportions of biologically active compounds in the blend produced by the flower would be constrained by pollinator-mediated selection (Raguso, 2008), and hence their variation should be more restricted than that of its biologically inactive compounds. In the third case, cross-pollination *via* a new pollinator would be possible, and could result in a speciation event, either by hybridization if the new pollinator also pollinates another *Ophrys* species, or in a new species if the newly attracted pollinator is only attracted to a subset of flowers producing this new bouquet. Ayasse *et al*. (2010) discussed these scenarios in the context of whether individual variation in odour bouquet is produced by chance followed by negative frequency-dependent selection of plants by their pollinators.

(*i*) *Speciation by hybridization*

We so far discussed the situation in which only one pollinator species pollinates each *Ophrys* species. A comparative analysis of pollinator networks among Euro-Mediterranean orchid species indeed indicated that *Ophrys* species are pollinated by a mean of 1.56 pollinator species, which is the lowest value for all orchid genera in this area (mean 7.44 pollinator species per orchid across genera, with a maximum of 158 pollinator species for the food-rewarding orchid *Neottia ovata*) (Joffard *et al*., 2019). A close examination of the currently available data on *Ophrys* pollinators (Claessens & Kleynen, 2011, 2016; Gaskett, 2011; Paulus, 2018; Joffard *et al*., 2019; Schatz *et al.* 2020) indicates that *Ophrys* species pollinated by several, usually congeneric, pollinator species are in fact groups of cryptic species. For example, six species of *Eucera* bees (Apidae) were reported as pollinators of *Ophrys bombyliflora* (Paulus, 2018), which has a wide circum-Mediterranean distribution. Such observations clearly require closer investigation especially if they were performed in different locations within the distribution area of this species. Subtle differences in flower morphology

and phenological variation suggest that *O. bombyliflora* includes a constellation of species (Delforge, 2005, 2016). The same holds true for *Ophrys tenthredinifera* and *O. lutea*. Despite this tendency for a higher number of pollinator species in widely distributed species (complexes), the large number of hybrids (more than 600 are currently described; Delforge, 2016) even among the 9–11 different clades identified by molecular systematics based on DNA sequences (e.g. Breitkopf *et al*., 2015; Bateman, 2018) must mean that other pollinators than their species-specific pollinator can sometimes visit a given *Ophrys* flower and transfer pollinia.

Postzygotic barriers seem rather rare in *Ophrys* [*e.g.* Scopece *et al*., 2007; but see Cortis *et al*. (2009) and Vereecken, Cozzolino & Schiestl (2010)], and hybridization is considered a source of evolutionary novelties that could ultimately lead to pollinator shifts and reproductive isolation (Cotrim *et al*., 2016). This scenario is illustrated nicely by a case study by Vereecken *et al*., (2010). These authors analysed hybridization between two species (*Ophrys lupercalis* and *O. arachnitiformis*) that use contrasting pollination strategies, and that belong to different clades according to the molecular phylogeny of Breitkopf *et al*. (2015). Males of *Andrena nigroaenea* (Hymenoptera: Andrenidae) pollinate *O. lupercalis* by introducing their abdomen into the stigmatic cavity of the flower and carry pollinia on their abdomen (abdominal position). Males of *Colletes cunicularius* (Hymenoptera, Colletidae) pollinate *O. arachnitiformis* by introducing their head into the stigmatic cavity of the flower and thus carry pollinia on their head (cephalic position). Experiments with the scent bouquet of their hybrids in Southern France found low attractiveness for either pollinator of the parent species, but it was attractive to a third species, *Andrena vaga*, which does not pollinate either of the parents. The scent bouquet of the hybrid includes odour compounds that are either absent from the parent species (two organic compounds), or expressed only at very low concentrations. Such evolutionary novelty in hybrids suggests that hybridization could have

contributed to the high pollinator diversification seen in *Ophrys*. However, the existence of these hybrids questions the generality of the assumed specificity between a given *Ophrys* species and its pollinator. Vereecken *et al*. (2010) published photographs of cross-copulation attempts, of *C. cunicularius* with *O. lupercalis* and *A. nigroaenea* with *O. arachnitiformis* (Fig. 3), showing that both pollinator species used both copulation positions (cephalic or abdominal). In this particular case, F1 hybrids between *O. lupercalis* and *O. arachnitiformis* were not fertile, and back-cross pollination between F1 hybrids and their parent species did not result in seed production. Further analyses showed that *O. arachnitiformis* was diploid, *O. lupercalis* was tetraploid and F1 hybrids were triploid, which might explain their sterility. Although in this case postzygotic isolation meant that hybridization failed to produce a new species, this example illustrates that speciation could occur assisted by the production of a new bouquet of organic compounds by the hybrid that attracts a new pollinator species.

Stökl *et al*. (2008) investigated pollinator syndromes in sympatric and allopatric populations of *Ophrys lupercalis* and *O. eleonorae* in Sardinia. *O. lupercalis* is widespread around the Mediterranean basin, whereas *O. eleonorae* is endemic to Sardinia and Corsica. These species are pollinated by *Andrena nigroaenea* and *Andrena morio* respectively, both using the abdominal copulation position. The authors used an integrated approach that combined morphological and chemical analyses of flowers, and GC-EAD and behavioural tests on pollinators. They also estimated hybrid indices of plants based on amplified fragment length polymorphism (AFLP) markers, and compared Sardinian plants with specimens from Majorca where only *O. lupercalis* is present, and from Greece where only *O. iricolor* is present. *O. iricolor*, to which *O. eleonorae* is vicariant, is a closely related taxon widespread in the eastern Mediterranean basin and is pollinated only by *Andrena morio*.

Morphological investigations of flowers indicated, in some Sardinian populations, the presence of phenotypes that had shapes and colours of the labellum intermediate between the

two parent species, and which were considered hybrids between *O. lupercalis* and *O. eleonorae*. As in other cases of flowers pollinated by *Andrena* species (Schiestl & Ayasse, 2002; Stökl *et al*., 2005, 2009), there was large overlap in the hydrocarbons released by flowers of the two *Ophrys* species. All the organic hydrocarbons were present in the bouquet of the two parent flowers and of their hybrids, albeit in significantly different proportions in 17 cases out of 24 for the parents. The proportions of organic hydrocarbons that were present in the bouquet of hybrids significantly differed from both parents in only one case out of 24. Behavioural experiments revealed that *ca*. 20% of the flowers from both parent species were attractive to both pollinators, and that this proportion was slightly higher (28%) for hybrids (Table 3).

Multivariate analyses (Principal Coordinate Analysis) on AFLP genotypes revealed that *O. lupercalis* individuals from Majorca clustered together and grouped with most *O. lupercalis* individuals from Sardinia and with some hybrids. *O. eleonorae* individuals from Sardinia and *O. iricolor* individuals from Greece clustered as two different groups. *O. eleonorae* individuals from Sardinia clustered with many hybrids and with a few Sardinian *O. lupercalis* individuals. Some hybrids were intermediate between Sardinian *O. eleonorae* and Greek *O. iricolor*. These results suggest the presence of introgression between the genomes of the two parent species in Sardinia presumably resulting from cross-pollination by non-specific pollinators. Introgression levels were much higher in *O. eleonorae*, which were genetically indistinguishable from most hybrids. The cluster formed by Sardinian *O. eleonorae* and most hybrids indicates how a parent species can be absorbed by a hybridogenetic taxon, eventually to form a new species. This example also demonstrates the important role of pollinators in the maintainance or the breakdown of reproductive isolation between *Ophrys* species. The observed genetic differences between *O. eleonorae* and *O. iricolor* confirm that they are closely related allopatric species. The designation of the Sardinian endemic *O. eleonorae* as a

separate species was proposed by Devillers & Devillers-Terschuren (1994), but not followed by Stökl *et al*. (2008) who considered Sardinian *O. iricolor* to be conspecifics of Greek *O. iricolor*. In a re-analysis of their data (Ayasse *et al*., 2010), the authors mention the existence of *O. eleonorae* but again do not consider it as separate to *O. iricolor*, despite the genetic differences they observed. This is a good example of the danger of unreliable taxonomic diagnosis: the direct consequence of the absorption of Sardinian *O. iricolor* by the hybrid *O. iricolor* × *O. lupercalis* would be the appearance of a new species, because *O. iricolor* persists in other parts of its range. However, the direct consequence of the absorption of the Sardinian endemic *O. eleonorae* by the hybrid *O. eleonorae* × *O. lupercalis* would be the substitution of the endemic *O. eleonorae* with a new species.

In our quest for the drivers of adaptive radiation in *Ophrys*, this example illustrates that speciation by hybridization could potentially contribute to an increase in species numbers. One of the parent species is being progressively absorbed by the hybrid, leading to the net result of two species (one new and one parent), instead of the two parent species. When this process of speciation by hybridization concerns metapopulations in restricted parts of the distribution ranges of the two parents, the absorbed parent species can maintain pure lineages in other parts of its range, with the net result being an increase in species number. We urge for the application of assignment tests of hybrids using genetic tools to confirm the parent species, as these are currently inferred too often from morphological similarities alone.

(*ii*) De novo *speciation*

The high inter-individual variation found in *Ophrys* species could allow the production of new blends of organic compounds that attract males of new pollinators that do not visit other *Ophrys* species. This could lead to *de novo* speciation events, which we illustrate below with two well-documented examples.

The pollination of *Ophrys speculum* by the wasp *Dasyscolia ciliata* (Hymenoptera: Scoliidae) is a well-known example of the *Ophrys* pollinator syndrome. It was for these two species that the first descriptions were published (Correvon & Pouyanne, 1916, 1923; Pouyanne, 1917) of the copulation attempts of male insects with the *Ophrys* flower labellum, and the potential role of odour in the attraction of their pollinator. The blend of organic compounds that attracts males and stimulates copulation behaviour was identified by Ayasse *et al*. (2003) using GC-EAD and behavioural experiments. Male antennae reacted to 10 components identified as saturated (ω-1)-hydroxy and (ω-1)-oxo acids, aldehydes and ethyl esters. Three components of the blend (9-oxodecanoic acid, 9-hydroxydecanoic acid and 7-hydroxyoctanoic acid) were identified for the first time in plants. Relative proportions of most of the GC-EAD-active compounds differed significantly between the orchid and females of its pollinator. However, the major component in the wasps (9-hydroxydecanoic acid) was also a major component of the active compounds in the orchid, where it is found at a three times larger concentration. Behavioural tests showed that *O. speculum* flowers were significantly more attractive to males than their own females; female dummies with a blend of synthetic organic compounds matching the blend of *O. speculum* flowers were more attractive than real females. Ayasse *et al*. (2003) suggest that the three times larger quantity of 9-hydroxydecanoic acid produced by the flowers was responsible for this difference, and suggest that the production of larger amounts of this attractant could be counter-selected in females due to the risk of attracting predators or brood parasites. This case study could perhaps explain why many apparently biologically inactive organic compounds are produced by *Ophrys* flowers. At some point, individuals of an ancestral species produced by chance one or several of the compounds that attract male *Dasyscolia ciliata*, allowing them to use this species as a new pollinator. We argue that the metabolic pathways leading to the production of organic compounds, will be under strong selection due to pollinator limitation. The

production by *O. speculum* of compounds that are unique in the plant kingdom is an indicator of the strength of this selection.

Our second example concern the molecular mechanisms behind reproductive isolation in *Ophrys sphegodes* and *O. archipelagi*, two sympatric and synchronic species that are phylogenetically closely related (e.g. Devillers & Devillers-Terschuren, 1994). *Ophrys sphegodes* is pollinated by *Andrena nigroaenea* (Hymenoptera: Andrenidae) and *O. archipelagi* by *Colletes cunicularius* (Hymenoptera: Colletidae). Despite their phylogenetic distance, males of these two solitary bees were attracted by the same family of organic compounds, i.e. n-alkanes and n-alkenes (Mant *et al*., 2005*b*). The major difference between floral odour in the two species was in the proportions of different n-alkenes (Xu *et al*., 2011; Sedeek *et al*., 2014). A field experiment investigating the transfer of stained pollinia found a perfect intra-specific match: of 15 and 21 flowers of *Ophrys sphegodes* and *O. archipelagi*, respectively, that received stained pollinia from insect pollinators, there was not a single inter-species transfer (Xu *et al*., 2011). Hand pollination showed that inter-specific crosses resulted in fruit set in all cases, and the viability of the seeds produced by these inter-specific crosses did not differ significantly from seeds produced from intra-specific crosses (Xu *et al*., 2011). These results suggest the presence of prezygotic isolation due to selective pollinator attraction in the maintenance of species barriers within this pair of closely related *Ophrys* species (Xu *et al*., 2011). Careful investigations of the volatile alkenes produced by these species revealed how the differential attraction of their pollinators is maintained. *Ophrys sphegodes* produces mostly 9- and 12-alkenes (i.e. with double bonds in positions 9 and 12, whereas *O. archipelagi* produces high levels of 7-alkene (i.e. with a single double bond in position 7). Two desaturase genes, *SAD2* and *SAD5*, that encode for stearoyl-acyl carrier protein desaturases (SADs), are responsible for either 9- and 12-alkene or 7-alkene production, respectively (Schlüter *et al*., 2011; Xu *et al*., 2012). SADs are soluble, nuclear-encoded,

plastid-localized proteins that act on an acyl group and catalyse the introduction of a double bond into saturated fatty acid precursors of alkenes (Schlüter & Schiestl, 2008; Schlüter *et al*., 2011); alkenes then are produced *via* elongation of these unsaturated fatty acids (Xu & Schlüter, 2015). The expression and enzymatic activity of *SAD2* typically is high in *O. sphegodes* and low in *O. archipelagi*, whereas the reverse is true for *SAD5*. The pollinator of *O. sphegodes* (*Andrena nigroaenea*) appears to be attracted more to 9- and 12-alkenes, whereas 7-alkenes reduce this attraction (Xu *et al*., 2012). Conversely, the pollinator of *Ophrys archipelagi* (*Colletes cunicularius*) is attracted by 7-alkenes; addition of 9- and 12-alkenes reduces this attraction (Xu *et al*., 2012). These pollinator preferences may thus have imposed divergent selection on odour bouquets of the two orchid species (Xu *et al*., 2012). Sedeek *et al*. (2016) showed how the enzymatic activity of the ancestral proto-*SAD5* protein generated both 7- and 9-alkenes; they proposed that restriction of *SAD5* to the production of 7-alkenes only, which is due to two amino-acid changes at a crucial position in the enzyme, resulted from pollinator-mediated selection. This example thus corresponds to the scenario of random crossing of peaks in the olfactory landscape by some *Ophrys sphegodes* individuals that over-expressed the ancestral *SAD5* enzyme. The resulting production of both 7- and 9-alkenes could have reduced attraction of *Andrena nigroaenea* while initiating attraction of *Colletes cunicularius*. Mutants that possessed the two amino-acid changes, and consequently that produced only 7-alkenes, would have realized the final step of *de novo* speciation by ensuring the integrity of the attraction of the new species-specific pollinator. In this example, speciation occurred through changes in expression of biologically active compounds.

Such *de novo* speciation events resulting from pollinator shift can only occur if several individual plants cross the same peak in the olfactory landscape at the same time and within the home range of the newly attracted pollinator, which might seem at first sight rather stringent conditions. However, the life-history strategies of *Ophrys* are particularly suited to

this. As previously mentioned, *Ophrys* individuals are long-lived (up to 20 years), which means that flowers with a particular blend of odours are present repeatedly within a population over a long time period, provided that flowers of a given plant produce the same blend of odours over its reproductive life. Data confirming the latter are not yet available. The strong competition for mnesic pollinators should select for the production of new blends of organic compounds, both by the generation of new molecules and by changes in the relative concentrations of existing compounds within the blend. Accordingly, the availability of new blends of organic compounds within a population should increase the chances that two or more *Ophrys* individuals would be cross-pollinated by a new pollinator species. Males of solitary bees may forage over distances of hundreds of meters to kilometers (Gathman & Tscharntke, 2002), increasing their probability of encountering individual plants producing similar bouquets. However, although the life-history strategies of *Ophrys* seem particularly suited for *de novo* speciation, the estimated diversification rate of between 4 and 8 lineages per million years (Breitkopf *et al*., 2015) still indicates that speciation remains a relatively rare event.

(*f*) *Directional selection of flower shape, colours and hairiness*

A new *Ophrys* species produced either by hybridization or by *de novo* speciation will be under both inter- and intra-specific competition. Inter-specific competition with parent species of the new taxon might take place if the new blend of attractive organic compounds is not sufficiently different from the odour bouquets of the parent(s). As the population of the new species increases in size, intra-specific competition between individual plants of the new taxon for the new pollinator will also increase. Besides the production of an original blend of organic compounds, *Ophrys* flowers can strengthen their attractiveness to their pollinator by increasing their morphological resemblance with the female. We expect strong directional

selection to result in increasingly accurate floral imitation of receptive females. The strong visual matching in some species is evident even to human observers (Fig. 4).

(*i*) *The role of flower colour, shape and pubescence in pollinator attraction*

Pollinators are attracted to *Ophrys* species first by analogues of sexual pheromones from a distance of *ca*. 5–10 m (Paulus 2006). At close proximity, visual signals trigger the landing of the pollinator on the labellum of the flower. *Ophrys* flowers hijack two categories of visual signals used by pollinators to detect their females: the colour of flowers visited by females looking for food, and the colour, shape and pubescence of the body of the female. These visual signals are produced by the upper part (perianth) and the lower part of the flower, respectively.

The first category was investigated using *Ophrys heldreichii* in a suite of experiments (Spaethe, Moser & Paulus, 2007; Streinzer, Paulus & Spaethe, 2009). The results showed a significant increase in pollinator attraction through an additive effect of visual signals (a large pinkish perianth) at close range (< 60 cm) to the olfactory cues produced by the labellum. Spaethe *et al*. (2007) proposed that selection may have favoured spectral resemblance between the pinkish perianth of the flowers of *O. heldreichii* and the reflectance of food plants visited by foraging females of the pollinator, *Eucera berlandi*. Streinzer *et al*. (2009) showed that the colour of the perianth is not the only visual cue used to locate flowers: the bees also use an achromatic visual channel that relies on information from green-sensitive photoreceptors for detection of flowers at closer range (< 30 cm). In attempt to generalize these results, Spaethe, Streinzer & Paulus (2010) found that perianth colours differed significantly between of *Ophrys* flowers pollinated by *Andrena* and *Eucerini* bees: green for *Andrena*-pollinated species and pink for *Eucerini*-pollinated species. They interpreted this as the consequence of differences in mate-locating strategies used by these two groups of bees

(more visual-based in *Eucerini* spp.). They suggest that the acquisition of a coloured perianth might carry a cost of potential pollen loss due to the attraction of a greater range of flower visitors, although this still has to be formally demonstrated. This genus-wide dimorphism in perianth colours according to pollinator fits nicely with the hypothesis of directional selection on *Ophrys* leading to the most efficient pollinator-attracting signals.

Co-variation between visual and olfactory signals could reduce the strength of selection on each signal, potentially invalidating our scenario of random crossing of peaks in the olfactory landscapes followed by directional selection on flower phenotypes. Vereecken & Schiestl (2009) investigated this in *Ophrys arachnitiformis*, which has two morphs differentiated by the colour of their perianths (either green or white). The frequency of each morph varies among populations, ranging from 100% green morphs along the Rhône to almost 100% white morphs in south-east France and northeast Spain (Vereecken & Schiestl, 2009). Studies of individual plants showed that colour morphs are stable in space and time, suggesting that this polymorphism has a genetic basis (Vereecken & Schiestl, 2009). The authors worked on two populations in which both morphs co-occur in southern France, and which were located *ca*. 20 km apart. They analysed the organic compounds that attracted the pollinator of *Ophrys arachnitiformis* (males of *Colletes cunicularius*) and found both relative and absolute amounts of active compounds were identical in the two morphs. Behavioural tests in the field using impregnated dummies showed that the olfactory signal was the only driver of pollinator attraction. Neither the presence of a perianth nor its colour influenced visitation rates of scented dummies by patrolling males of *C. cunicularius*. Variation in visual signals thus does seem to be decoupled from olfactory signals at least in this case. However, this study was focused on variation in perianth colour, and future work should also investigate the effects of variation in labellum colour and olfactory signals on pollinator attraction. Sedeek *et al*. (2014) investigated the genomic structure of four closely related *Ophrys* species. Using genomic

scans for F_{ST} outliers, the authors detected only a few markers that are highly differentiated among these four species. In addition, the authors found elevated linkage disequilibrium among these few repeated outlier loci and that among-outlier linkage disequilibrium increased with species divergence. Accordingly, Sedeek *et al*. (2014) suggested that the few repeated outlier loci identified probably stem from a few common genomic regions. However, as the function of most of these F_{ST} outlier loci cannot currently be inferred, questions regarding possible linkage disequilibrium (and physical proximity) between those loci and genes coding for olfactory and visual signals still cannot properly be addressed. Recent reviews of intra-specific variation of floral odour and colour–odour associations in the orchid family have highlighted the existence of broad variation depending on biochemical constraints, pollination strategy and habitat requirements (Delle-Vedove, Schatz & Dufaÿ, 2017; Dormont *et al*., 2019)

The shape, colour and pubescence of the lower parts of the *Ophrys* flower are the second category of signals hijacked by the orchid to mimic the female of their pollinator. Rakosy *et al*. (2017) investigated how the shape of the labellum affects pollinators. By using 3D techniques, these authors showed that several crucial coordinates of the labellum of *Ophrys leochroma* aligned their pollinators (male *Eucera kullenbergi*) for efficient pollination. Using similar techniques, Sedeek *et al*. (2014) showed that the position of the viscidium (the adhesive disk where the caudicule of the pollinaria is inserted) differed on flowers of four related *Ophrys* species, suggesting that their individual pollinators adopt different positions during their copulation attempts. Devillers & Devillers-Terchuren (2000) previously documented the essential role of the labellum in ensuring effective pollination by mechanically guiding pollinators towards the reproductive structures of the flower, and by offering them gripping points on the labellum similar to those on the female body. Rakosy *et al*. (2017) showed that pollinators were significantly less effective in interacting with

experimentally manipulated flowers with a shape that was altered to resemble *Ophrys* species pollinated by other hymenopteran genera. They proposed the existence of mechanically active and inactive components of labellum morphology, under pollinator-mediated selection similarly to biologically active and inactive compounds present in the olfactory signals. Accordingly, mechanically active components of the flower form could reflect adaptations to interaction with particular pollinator groups, while inactive components could vary more freely. Such results are promising and valuable insights into the mechanisms driving morphological diversification of the functionally different components of *Ophrys* flowers.

A classification of the shape, colour and pubescence of the lower parts of the *Ophrys* flower was proposed by Paulus (2006), who distinguished three classes of *Ophrys* flowers according to the intensity of their match with the body of the female of their pollinator. In the first class, there is a perfect match between the shape and the colour of the flower and its pollinator. This is the case for *Ophrys speculum*, where "the blue mirror of the labellum imitates the blue iridescence of the female's wings and the reddish-brown hair-like structures on the labellum imitate the red body hairs of the female wasp" (Paulus, 2006, p. 320) and "the reddish brown colour of the hairs of the labellum matches almost miraculously the color of the body pubescence of the female wasp" (Paulus, 2006, p. 320). The pollinator of *O. speculum* is males of the wasp *Dasyscolia ciliata ciliata* in the western Mediterranean. In the eastern part of its range, the vicariant of *O. speculum* (*Ophrys eos*) is pollinated by the vicariant wasp *Dasyscolia ciliata araratensis*, in which females have a dark-brown body pubescence. Accordingly, the pubescence of the margins of the labellum of the vicariant *Ophrys eos* is conspicuously darker (Paulus, 2006). In the second class of Paulus (2006), the similarity between the flower and the body of the female of its pollinator is less accurate. Among the examples proposed by Paulus (2006), we select the flowers of *Ophrys kotschyi* and *O. cretica* that are both pollinated by the bee *Melecta tuberculata* (Hymenoptera,

Melectidae). These two species belong to two different lineages, the clade of *O. umbilicata* (*O. kotschy*i) and the clade of *O. holoserica* (*O. cretica*) (Fig. 1), as demonstrated by the analysis of supposedly neutral intergene spacer regions (nrITSs) and a choroplast intergenic spacer region (*Rrn5-Trn*) (Sramkó, Gulyás & Molnár, 2011). Despite their distant relatedness, flowers of both species show marked convergence in colours: their green perianth is tinged with pink, the bottom of the stigmatic cavity is white (which is very uncommon in *Ophrys* flowers), the white, complex H-shaped speculum of the labellum is large and shows a strong contrast with the dark-brown background colour of the labellum, and the upper part of the labellum has large white margins. These colour patterns are obvious imitations of the black and white patches on the body of females of *Melecta tuberculata*. *Ophrys* flowers of the third class of Paulus (2006) imitate only the background colouration of the females of their pollinator. Most representatives of this category belong to two clades subject to ongoing divergence: the *O. fusca* clade and the *O. sphegodes* clade (see Fig. 1). An example of this class is the labellum of *O. sphegodes*, which matches the reddish brown or grey-brown colour of the female of *Andrena nigroaenea*, whereas the deep dark blackish brown of *O. incubacea* matches the dark body colour of *Andrena morio*.

These three classes were considered as evidence of the role of the visual signalling in the mating behaviour of their respective pollinators by Paulus (2006), from 'very important' (Class 1) to 'not important' (Class 3). Here, we suggest an alternative, more parsimonious explanation that is rooted in the evolutionary history of the different clades of *Ophrys*. We propose that these three classes correspond to a gradient in the evolution towards an ever-more-perfect match between *Ophrys* flowers and the visual signals associated with mate recognition by *Ophrys* pollinators. This evolution reflects directional selection based on the higher reproductive success of flowers that display the best visual signals. Based on the ages of the different clades belonging to each of these three categories according to the dated

phylogeny of Breitkopf *et al*. (2015) (Fig. 1), *Ophrys speculum*, which is Class 1, is among the most ancient clades. The clades of *Ophrys fusca and O. sphegodes*, to which belong the species of the third category, are much younger, and are clades in which there is ongoing rapid speciation (Fig. 1). Thus we suggest that visual signals have not yet been perfected in these younger groups by directional selection.

All molecular phylogenies to date concur that floral morphology leading to copulation attempts in the abdominal position is a synapomorphy shared by all the species belonging to the paraphyletic *Pseudophrys* former subgenus (e.g. Breitkopf *et al*., 2015; Bateman *et al*., 2018*a*). To explain how this synapomorphy arose, we assume the appearance of a deviant morphology in flowers of species belonging to one of the basic clades of the phylogeny (Fig. 1). This new morphological type arising either by mutations of genes controlling flower morphogenesis and flower hairiness, or by hybridization between taxa from distant clades with contrasting morphologies, or by a combination of these two, would have by chance more efficiently targeted the abdomen of the pollinator towards the retinaculum and the stigmatic cavity compared to flowers on which males moved randomly. The higher reproductive success of those flowers could have led to the spread of this key innovation, and favoured rapid radiation in this group by *de novo* speciation and/or hybridization, as demonstrated by phylogenomic analyses (Breitkopf *et al*., 2015; Joffard *et al*., 2020).

(*ii*) *Directional selection on flowers of recently separated species*

Whether directional selection acts on flowers after speciation can be tested on pairs of species that have recently separated. The *O. insectifera* clade that diverged early within *Ophrys* according to the dated molecular phylogeny of Breitkopf *et al*. (2015) (Fig. 1) seems an excellent candidate to test this assumption. In this clade, the widely distributed *O. insectifera* is associated with two endemic seemingly recently separated vicariants (Devey *et al*., 2008;

Triponez *et al*., 2013; Breitkopf *et al*., 2015). The most parsimonious hypothesis is that the most widespread species is the parent of its endemic vicariants, and indeed it is sympatric with populations of the two vicariants: *O. subinsectifera* which is restricted to the southern foothills of the central and eastern Pyrenees, and *O. aymoninii* which is endemic to the calcareous part of Massif Central in southern France. These three species each have a distinct hymenopteran pollinator (a wasp, solitary bee and sawfly) of contrasting size (Table 4, Fig. 5). These pollinators belong to three different families, which is unique within the *Ophrys* genus. Molecular insights from chloroplast DNA sequencing and AFLP genotyping indicate recent diversification in the *O. insectifera* clade, which may have been further obscured by migration and admixture across the European continent (Triponez *et al*., 2013). Genetic results indicate weak but noticeable phylogeographic clustering that correlates only partially with species limits. Moreover, several isolated haplotypes and genetic clusters were reported for *O. insectifera* in central and southeastern Europe, which might be an indication of ongoing speciation in this clade (Triponez *et al*., 2013). The flowers of the three species show remarkable similarities in shape and colouration, notably an elongated labellum with a central blue-grey horizontal bar (Fig. 5). They differ from other *Ophrys* flowers in the form of the labellum, which has three well-delineated lobes (two laterally and one basally, the latter being subdivided in two parts), in the elongation and lateral constriction of the petals, and in the location of the pseudo-eyes on the labellum rather than on the stigmatic cavity (Devillers & Devillers-Terschuren, 1994). These flowers show a remarkable convergence with an insect body with the reduced petals mimicking the antennae, the stigmatic cavity mimicking the head, the anterior lobes mimicking the wings, and the rest of the labellum mimicking the thorax and abdomen (Fig. 5).

Careful examination and measurements of the flowers reveals differences among these three taxa (Fig. 5). The labellum of *O. insectifera* is usually devoid of a yellow border,

whereas flowers of *O. subinsectifera* have a yellow outer border ranging from 1 to 1.5 mm in width, while the yellow outer border of *O. aymoninii* ranges from 1 to 2.5 mm (Triponez *et al*., 2013). The petals of *O. subinsectifera* are shorter (2–4 mm) than those of the two other species (4–7 mm). Importantly, the length of the labellum of the three species is significantly different (*ca*. 12 mm for *O. insectifera*, 9.5 mm for *O. aymoninii* and 6.5 mm for *O. subinsectifera*; Triponez *et al*., 2013). This difference in labellum length matches the non-overlapping body lengths of the three pollinators (Table 5).

Following our speciation scenario, new pollinators could have been attracted by locally divergent individuals of *O. insectifera* that randomly crossed a peak in their olfactory landscape to escape intra-specific competition for limited, mnesic pollinators. Comparison of labellum extracts from *O. insectifera* and *O. aymoninii* showed that of the five organic compounds found to be GC-EAD active in males of *Andrena combinata* (the pollinator of *O. aymoninii)*, four are present in significantly higher relative amounts in *O. aymoninii* (Gervasi *et al*., 2017). Application of these four compounds to flowers of *O. insectifera* triggered the approach and landing of males of *A. combinata* (Gervasi *et al*., 2017). No pollinia transfer between these two taxa was recorded in a field experiment using artificially stained pollinia, and pre- and postzygotic barriers are absent, as shown by the success of artificial inter-specific pollinia transfer that led to fruit set and seedling development (Gervasi *et al*., 2017).

Inter-specific crossing between *O. insectifera* and *O. aymoninii* thus seems prevented by differences in floral scents that attract specific pollinators. Our speciation scenario then suggests that directional selection on flower traits would have taken place to reinforce attractiveness to pollinators. The observed differences in flower shape, colours and size meet this prediction. The difference in labellum length likely would be a decisive character maintaining isolation between the two taxa by limiting or preventing cross-pollination. Isolation between *O. insectifera* and *O. subinsectifera* seems less advanced along this path to

speciation. Flowers of *O. subinsectifera* emit three aldehydes that are absent from the bouquets of the two other species (Joffard *et al*., 2016). Unfortunately, no data are available on their physiological activity or on the presence of other pre- or postzygotic isolation mechanisms. However, careful observations by Paulus (2017) document that males of *Sterictophora gastrica* (the pollinator of *O. subinsectifera*) are also attracted by sympatric individuals of *O. insectifera*, suggesting incomplete olfactory isolation. When *O. insectifera* is in sympatry with the two other *Ophrys* species, very rare hybrids are observed in both cases (B. Schatz, unpublished data).

Nonetheless, directional selection leading to differences in flower traits has already taken place, leading to differentiation of *O. subinsectifera* flower size, shape and colours. The attraction of males of *Argogorytes* spp. to *O. aymoninii* has not been reported to date; copulation attempts would be unlikely due to the difference in size between the large pollinator and the small labellum of the flower. The pollinator of *O. subinsectifera* is a sawfly, which is unique in *Ophrys*, and males use an unusual posture during pollinia transfer. Rather than trying to perform a copulation attempt with the flower either in a cephalic or abdominal position, males of *Sterictophora gastrica* move frenetically on the flower and pick up pollinia on their thorax, legs or the side of their abdomen (Souche, 2007; Geniez, Schatz & Escudié, 2016; Paulus, 2017). This frenetic behaviour corresponds to the prediction of Ågren *et al*. (1984, p. 24), who wrote that "a chemical stimulus without the proper tactile stimulation only results in undirected crawling… of the males". This argues in favour of a very recent differentiation of *O. subinsectifera*, and we suggest that progressive evolution of flower morphology, and hairiness could increase the efficiency of pollination of *O. subinsectifera* flowers by *Sterictophora gastrica*. The behaviour of males of *Sterictophora gastrica* thus provides insights into the evolution of the morphology of flowers pollinated by males attempting to copulate using the abdominal position.

Altogether, the differences in flower shape, size and colours in the *O. insectifera* clade thus provide us with a glimpse into selection on flower morphology after a pollinator shift (Fig. 6). Future experimental work should focus on: (1) testing for differences in flower odour and morphology among *O. insectifera* individuals that are either in sympatry or in allopatry with both vicariants, to phenotype hybrids, and to investigate whether flower shape, and especially labellum length, favours or impedes pollinia removal in con- and inter-specific cross-pollination experiments; (2) investigating habitat requirements of the three taxa in relation to those of their pollinators; (3) testing whether the different genetic clusters found in *Ophrys insectifera* correspond to ongoing speciation towards new endemics using genotypic and phenotypic data together with data on habitat requirements and pollinator identity; (4) investigating whether the two main pollinators of *O. insectifera* (equating roughly to one in the southern part of its range and the other in the northern) have induced pollinator-mediated selection on the olfactory signals emitted by the flowers.

(*iii*) *Directional selection on flowers of the same species that use two different pollinators*

A recent review of orchid–pollinator systems in the Euro-Mediterranean region found several potential cases in which one *Ophrys* species is pollinated by two or more pollinators (Joffard *et al*., 2019). Investigations considering main and secondary pollinators are still rare, precluding reliable separation of these two categories. We tentatively generalize that the use of two different pollinator species may be found in *Ophrys* species either with a wide, continuous distribution range (as in *Ophrys insectifera*) or with a discontinuous range such as in taxa isolated on islands. Such insular situations provide a unique opportunity to investigate how allopatric speciation interacts with pollinator shifts. Different metapopulation lineages of the same parent taxon attracting two or more pollinator species could move towards speciation through directional selection on species-specific pollinator signals. Such speciation

events will be facilitated by the disruption of gene flow between incipient species in allopatry. Accordingly, we expect to find spatially disjunct metapopulation lineages at various stages of the speciation process, which begins by the attraction of new pollinators by morphologically similar individuals. Such situations of ongoing speciation illustrate the utility of the unified species definition, according to which species diversification is a continuous process. Breitkopf *et al*. (2013) documented that in the Italian peninsula, an Adriatic population of *O. sphegodes* exclusively attracted *Andrena nigroaenea*, whereas a Tyrrhenian *O. sphegodes* population preferentially attracted *A. bimaculata*. These populations are separated by > 200 km and by the Apennine Mountains. This pollinator shift was associated with significant differences in scent-component proportions of the orchids. However, using neutral markers (AFLP), the authors did not detect significant intra-specific genetic structuring between these two populations. These results suggest that these two populations may have reached an early stage of divergence and are adapting to different pollinator species.

Allopatric speciation is evident on the five main islands of the Mediterranean Sea (Sicily, Sardinia, Corsica, Cyprus, Crete), which all have several endemic and sometimes closely related species (e.g. Delforge, 2016). The presence of endemic species is also known on some other islands such as Malta and Hvar, but the main hotspot of *Ophrys* endemicity is the archipelago of Aegean Sea (e.g. Karpathos, Andros, Astypalea and Rhodes) where geographic isolation coincides with diverse environments (Delforge, 2016; Schatz, 2017). The presence of *Ophrys* species is strongly limited on very small islands where a sustainable, long-term presence of their pollinators is uncertain (Schatz, 2017). However, there are numerous situations in which closely related *Ophrys* species are present on a few islands: they could represent an ideal experimental arena to test our scenario of speciation by pollinator shift followed by the progressive evolution of pollinator-attraction signals.

In addition to differences in allopatric pollinators, some studies report the use of several related pollinator species in the same locality. Paulus (2018) mentions pollination by an additional two species (*Andrena thoracica* in France and *A. limata* in SW Germany) closely related to the main pollinator of *Ophrys sphegodes* (*A. nigroaenea*). He suggests that sharing of the pollination function may occur when there is no competition among *Ophrys* species for a given pollinator, implying some control over the efficiency of its pollinator(s) by the plant. To support this interesting hypothesis, Paulus (2018) reports a case study on *Ophrys fuciflora* in which flowers were visited almost at the same rate by the main pollinator *Eucera longicornis* and by the closely related *E. nigrescens*. The latter species revisits *Ophrys* flowers much more rarely, probably due to imperfect correspondence between the bouquet of organic compounds emitted by the flowers and the sexual pheromone of *E. nigrescens* virgin females. Paulus (2018) suggests that mnesic males of *E. nigrescens* associate this imperfect odour bouquet with a vain copulation attempt, and avoid further contacts with the flowers of *O. fuciflora* after their first trial. We could also interpret this avoidance by suggesting that the variations in the odour bouquet produced by flowers of *O. fuciflora* are not sufficiently different for *E. nigrescens* males, so that they consider all other flowers as already visited after a first copulation attempt. *E. nigrescens* males thus 'steal' pollinia instead of performing pollination. Paulus (2018) points out that the flowering period of *O. fuciflora* coincides with the emergence of fresh males of *E. longicornis* at a time when *E. nigrescens* males (which emerged earlier) are becoming rarer and older. The floral phenology might thus be an adaptation to reduce wastage of pollinia by the less-effective pollinator. We can expect to see over time an ever-closer match between a plant and its best pollinator species, involving directional selection on the flowering period, floral morphology and the organic compounds emitted by the flower.

(*iv*) *Directional selection on species sharing the same pollinator*

When two species of *Ophrys* species share the same pollinator, we anticipate directional selection leading to convergence in some floral traits to secure pollinator attraction, but divergent selection on other floral traits to limit or avoid hybridization. One example of these antagonistic processes has been reported for *Ophrys normanii–O. chestermanii* (Gögler *et al*., 2009, 2011, 2015). Both species are endemic to Sardinia where sympatric and allopatric populations coexist, and they share the same pollinator, male *Bombus vestalis*, which removes the pollinia on his head *via* copulation attempts in the cephalic position. These two orchid species are paraphyletic according to genetic analyses using both AFLP and plastid markers (Gögler *et al*., 2009), in agreement with the dated molecular phylogeny of Breitkopf *et al*. (2015). They are considered to belong to relatively distant lineages, *O. normanii* being a member of the rather primitive *O. tenthredinifera* clade, whereas *O. chestermanii* belongs to the more recent clade *Ophrys holoserica* clade (Breitkopf *et al*., 2015; Fig. 1). The flowers of these species show a remarkable convergence in shape, colour and hairiness. In particular, they both have black stigmatic cavities and black basic fields, unlike all other species in their respective clades.

Odourless dummy bees impregnated with labellum extracts from both species attract males of *Bombus vestalis* that perform copulation attempts in flight-cage experiments (Gögler *et al*., 2009). Using GC-MS and GC-EAD, Gögler *et al*. (2011) found that the bouquet of organic compounds produced by the flowers of both species were remarkably similar to the pheromones of virgin females of *Bombus vestalis*. Given this similarity of the floral odour bouquet, the overlap of their flowering periods and the co-occurrence of both species in sympatric populations, hybridization between these taxa is expected. Genetic assignment of 99 individuals of *O. chestermanii* and 65 individuals of *O. normanii* failed to detect gene flow between these two species (Gögler *et al*., 2009). Moreover, inter-specific cross-pollination

experiments showed that 11 out of 15 crosses with *O. chestermanii* as the pollen donor resulted in fruit set, and 13 out of 15 crosses with *O. normanii* as the pollen donor resulted in fruit set. In all but one case, seeds of these cross-pollination combinations germinated, and produced 100–150 plantlets from each combination after 18 months (Gögler *et al*., 2009).

Given the absence of gene flow and of postzygotic isolation despite remarkable similarity in the composition of the odour bouquet emitted by the flowers, we expect the existence of an efficient prezygotic isolation mechanism. Careful measurements of flower morphology were performed on individuals from both species sampled in sympatric and allopatric populations (20 flowers/population; 8–20 km between sympatric and allopatric populations). These measurements concerned three floral traits involved in the pollination process: the width and the height of the stigmatic cavity and the length of the pollinaria, and a fourth trait, the length of the central outer tepal, which is not involved in the pollination syndrome, as a control. The results indicate unambiguously that the values of the three floral traits associated with pollination were significantly less similar between the two species in the population in sympatry compared to their values in allopatric populations, whereas no difference was detected for the control trait (Gögler et al. 2015). The stigmatic cavity of *O. chestermannii* individuals was smaller in the population where both species were in sympatry. The pollinaria of *O. normannii* are significantly longer than those of *O. chestermannii*. This led Gögler *et al*. (2015) to postulate that the long pollinaria of *O. normannii* fixed to the head of males of *B. vestalis* would not fit into the smaller stigmatic cavity of *O. chestermannii*; this was confirmed by video observations of copulation attempts. These differences in floral traits associated with pollination in the sympatric population relative to the allopatric population suggest selection on characters leading to prezygotic isolation between these two phylogenetically distinct taxa, nicely fitting the hypothesis of divergent selection on some floral traits to avoid hybridization.

Thus directional selection can lead not only to convergent evolution towards flowers with similar morphology, but also to mechanisms that allow prezygotic isolation by mechanically preventing cross-pollination between flowers with similar visual attraction signals for the pollinator. The presumed character divergence in sympatric populations suggests that prezygotic mechanisms will be under strong selection.

(2) Exploitation of protandry and directional selection on flowering period

Most *Ophrys* pollinators (adult male solitary bees) emerge (up to a few days) before their females (protandry) (Alcock *et al*., 1978; Hutchings *et al*., 2018). In insects in which females mate only once and males are capable of multiple matings, male development time is under strong intra-sexual selection, as those males that are active and sexually mature at the time of virgin female emergence will maximize their mating success (Wiklund & Fagerström, 1977, Parker & Courtney, 1983; Zonneveld & Metz, 1991). Accordingly, it could be argued that plants using pollination by sexual swindling should be in bloom when male competition is at a peak, but when there is a relative deficit of virgin females (Nilsson, 1992). This appealing hypothesis is contradicted by the longevity of flowers in *Ophrys*, although we acknowledge the limited availability of data on this critical biological parameter. Francisco & Ascensão (2013) mention periods of anthesis of 6–8 days and 9–12 days for *Ophrys bombyliflora* and *O. tenthdedinifera*, respectively, whereas Neiland & Wilcock (1995) observed that unpollinated flowers of *O. tenthredenifera* and *O. arachnitiformis* remained viable in the field for three weeks or more. As each *Ophrys* inflorescence carries several flowers that open successively, albeit with a slight overlap between subsequent flowers, a given *Ophrys* individual with a mean of four flowers should present at least one attractive flower continuously for *ca*. 4–6 weeks. Note however that the attractiveness of each flower may change with time. It appears from this long time window that the plants are not able to match

exactly the time window of emergence of their pollinators, although this could be due to unpredictability in space and time of the emergence of pollinators. Although it is possible to calculate the required degree-day accumulation for solitary bee emergence (e.g. White, Son & Park, 2009), micro- and meso-climatic heterogeneity can lead to a relatively wide time window during which emergence occurs (Forrest & Thomson, 2011). Moreover, holometabolous insects can delay their emergence according to ambient weather conditions for days or even weeks (e.g. Hermann *et al*., 2016). Finally, historic data of both the flowering dates of *Ophrys sphegodes* and the flight periods of its pollinator (*Andrena nigroaenea*) over more than 350 years provide interesting and rare evidence that *Ophrys* flowering periods have become increasingly imperfectly matched to the phenology of their pollinators under the effects of global warming (Hutchings *et al*., 2018). As the rate of development of these bees has increased faster with increasing ambient temperature than that of *Ophrys* (Robbit *et al*., 2014), the interval between the start of the males' flight period and the flowering time of *Ophrys* has increased. The interval between male and female emergence has decreased with climate warming, thus the proportion of females in the population during the *Ophrys* flowering period has also increased, which is likely to lower the attractiveness of flowers to naïve males (Robbit *et al*., 2014). Current rates of climate warming indicate a progressive time shift between pollinator emergence and *Ophrys* flowering, decreasing the plants' chances of pollination.

Whatever its reason, the long period during which a given *Ophrys* individual has flowers at anthesis might facilitate the random crossing of olfactory peaks leading to the attraction of a new species of pollinator. Adults of a given species of solitary bee are usually on the wing for only 8–12 weeks, but different species emerge at different times. For example, at least one *Andrena* species is on the wing in the UK for the period between February and September (Williams & Edwards, 2012; Else & Edwards, 2018). Accordingly,

directional selection could act on subsets of plants that attract a new pollinator species and interbreed, resulting in a more precise match between the flowering period of this newly formed *Ophrys* taxon and the flight period of its pollinator. The end result of such directional selection would be staggered flowering periods of closely related *Ophrys* species that attract different species of closely related pollinators.

A good example is provided by the west-Mediterranean *Ophrys fusca* clade pollinated by males of solitary bees from the genus *Andrena* (Paulus, 2006). In this group, which is considered as recent in origin by molecular phylogenies (Breitkopf *et al*., 2015; Joffard *et al*., 2020; Fig. 1), morphological differentiation seems rather weak, whereas temporal segregation of the flowering period of syntopic species is well established. Table 5 shows the flowering periods of a suite of very similar species of the *Ophrys fusca* clade belonging to the *Ophrys attaviria* group (*sensu* Delforge, 2016) that are all found in Crete. All these species are pollinated by different *Andrena* species and their flowering period is remarkably staggered from the beginning of January to the end of May. Only one of these species (*Ophrys cinereophila*) is widely distributed in Crete and in the Eastern part of the Mediterranean basin; the others are all very rare and restricted to different calcareous mountain massifs in Crete. This fits well an incipient speciation scenario in which species differentiation based on attraction of a new pollinator within a metapopulation is followed by a directional selection on flowering period leading to progressive divergence from the parent taxon. Morphological divergence has not yet taken place. We anticipate that interbreeding between individuals that have attracted by chance a new pollinator will only occur locally, such that newly differentiated taxa will have smaller distribution ranges. Such narrow distribution ranges might thus be used to identify recent speciation events.

(3) Pollinator repulsion after pollination

Pollination in *Ophrys*, i.e. the depositing of pollinia on the stigmatic cavity of a flower, is often also associated with the removal of a pollinia from this newly pollinated flower by the pollinator (e.g. Schiestl & Ayasse, 2001). The prolonged maintenance of the attractiveness of a pollinated flower will have a low additional reproductive benefit to the plant. We thus expect the selection of mechanisms limiting the attractiveness of pollinated flowers, especially when other, unpollinated flowers at anthesis are present on the same plant.

(*a*) *Emission of anti-aphrodisiac compounds*

Recently pollinated *Ophrys* flowers emit anti-aphrodisiac compounds that are repulsive to pollinator males, similar to pheromones that are produced by mated hymenopteran females to avoid further courtships. Schiestl *et al*. (1997) recorded changes in the olfactory signals produced by pollinated flowers, both in terms of decreased total amounts of organic compounds produced and changes in their composition, with some repellent compounds reaching high relative proportions. The key role of the fatty ester all-trans-farnesyl hexanoate was demonstrated in the repulsion of pollinators by Schiestl & Ayasse (2001) who compared the organic compounds present in the air immediately above the flower and in labellum extracts of unpollinated flowers and of flowers 2–4 days after pollination. They found a decrease, albeit non-significant, of electrophysiologically active compounds in the labellum extracts of pollinated flowers of *O. sphegodes*, but, more importantly, a significant increase in both the absolute and relative amounts of all-trans-farnesyl hexanoate in the headspace of pollinated flowers. This fatty ester produced by pollinated flowers is also emitted by females of its pollinator (the solitary bee *Andrena nigroaenea*) after mating (Schiestl & Ayasse, 2000); females use this ester to line their brood cells. In bioassays, bee dummies impregnated with attractive scents from unmated females combined with farnesyl hexanoate elicit

significantly fewer approach and copulation attempts by males than dummies impregnated with attractive scents only (Schiestl & Ayasse, 2000). Similarly, presentation of flowers of *Ophrys sphegodes* artificially scented with an amount of farnesyl hexanoate equal to the increased amount after pollination reduced the number of pollination attempts by male bees and were thus significantly less attractive than flowers treated with solvent only (Schiestl & Ayasse, 2001). These authors suggested that emission of this ester by pollinated flowers is a signal that will increase pollinator visits to other flowers on the same inflorescence. However, successive visits could expose the plant to geitonogamy, i.*e.* the pollination of a flower by another flower of the same inflorescence, which will have similar consequences to autogamy in terms of decreased seed production and viability. To counter these deleterious outcomes of geitonogamy, careful observations by Ayasse *et al*. (2000) revealed a subtle mechanism. In this species, pollination can be achieved only if the pollinia carried by the bee on its head from one flower have bent forward to contact the stigmatic surface of a subsequent flower visited. The time for complete bending of pollinia (161.9 ± 10.1 s, $N = 10$; Claessens & Kleynen, 2011) is longer than the total time spent by males visiting the first, second, and third flower of an inflorescence, and on moving between these flowers (Ayasse *et al*., 2000). This exemplary study constitutes the only documented example in *Ophrys* of modification of signals between the flower and its pollinator induced by pollination, but it provides many insights into the eco-evolutionary dynamics between *Ophrys* and their pollinators.

(*b*) *Repulsion by learning visual signals*

Pollinator attraction involves a combination of olfactory and visual signals, and repulsion of pollinators by pollinated *Ophrys* flowers seems also both channel types. Schiestl *et al*. (1997) reported that the colours of pollinated flowers of *Ophrys sphegodes* faded within three days following pollination. Gaskett (2011) suggested that variation in floral shape could impair

pollinator learning avoidance in the same way that variation in scent hinders this process. Using an elegant experimental approach, Stejskal *et al*. (2015) investigated the function of the complex patterns on the labellum (the speculum) of the flowers of *Ophrys heldreichii*. These are in the form of complex and variable whitish patterns on a dark-brown background. Field observations revealed that after a copulation attempt, males of its pollinators (the bee *Eucera berlandi*) fly at close distance in front of the flower for around a minute. The authors interpreted this behaviour as scanning by the bee of the pattern on the speculum to avoid further copulation attempts with the same flower, and even with flowers on the same inflorescence. Comparisons of labellum patterns revealed that patterns within inflorescences are more similar than they are with patterns on other conspecific plants. The authors hypothesized that the function of these patterns on the speculum for the plant is the avoidance of geitonogamy. According to this hypothesis, this mechanism based on pattern learning should be less efficient from the plant's perspective than the emission of anti-aphrodisiac compounds from a pollinated flower, because with pattern learning pollinators are deterred from visiting other flowers on the same inflorescence. To test the ability of pollinators to learn labellum patterns, Stejskal *et al*. (2015) trained honeybees (*Apis mellifera*) and showed that trained individuals were able to discriminate among labellum patterns from different plants, but not among labellum patterns from the same inflorescence, supporting the hypothesis that variable labellum patterns in *O. heldreichii* are involved in flower–pollinator communication, likely allowing these plants to avoid geitonogamy.

IV. THE POLLINATOR VIEWPOINT: DECEPTION OR BENEFIT?

Almost all *Ophrys* species use pollination by sexual swindling, i.e. flowers mimic signals produced by virgin females of their pollinators to elicit copulation attempts by males that ensure pollinia transfer. Observations of this behaviour led to the introduction in the literature

of the concept of 'pollination by sexual deceit', according to which the plant is considered as parasitizing male sexual behaviour (e.g. Vereecken, 2009). From the perspective of the pollinator, costs are indeed evident. Duped males attracted to *Ophrys* by the manipulation of combined olfactory, visual and tactile signals lose time and energy in copulation attempts with flowers. Some data exist on the duration of copulation attempt by male pollinators. For the pair *Ophrys leocchroma–Eucera kullenbergi*, Rakosy *et al*. (2017) found a significant positive relationhip between the duration of the copulation attempts (ranging from 5 to 60s) and its efficiency as estimated by the number of massulae deposited in the stigmatic cavity. For *Ophrys galilaea–Lasioglossum marginatum*, Machaka-Houri *et al*. (2018) recorded a mean duration of 14 s and for *Ophrys heldreichii–Eucera berlandi*, Stejskal *et al*. (2015) reported a median duration of 5.7 s (range 0.7–39.9 s). This duration is consistent with the length of time required for the successful transfer of sperm in honeybees (a few seconds; Koeniger & Koeniger, 1991; Winston, 1991) or in stingless bees (less than a minute; Engels & Engels, 1988). Copulation entails costs (Brown & Baer, 2005) in terms of increased exposure to predation because copulating pairs have greatly reduced mobility (Brown & Baer, 2005), and are more conspicuous. In two Australian *Cryptostylis* orchids that use a similar sexual-swindling pollination syndrome, males of their shared pollinator (the wasp *Lissopimpla excelsa*, Ichneumonidae) not only attempt to copulate with flowers mimicking their females, but also ejaculate onto the flowers, as demonstrated by the presence of sperm in the stigmatic cavity (Gaskett, Winnick & Herberstein, 2008). To the best of our knowledge, the presence of insect sperm on the labellum of *Ophrys* flowers was mentioned only by Willmer (2011), without references for her primary sources. This certainly deserves attention. The antennal grooming or visual scanning of the patterns on the speculum that record olfactory or visual signals, respectively (Paulus, 2006) that take place after a copulation attempt with an *Ophrys* flower correspond to the normal end of mating behaviour for

hymenopteran males that record the identity of their partner. This behavioural sequence suggests that males are unable to differentiate between copulation with mates and with floral lures (Willmer, 2011).

Johnson & Schiestl (2016) suggested that the evolution of counter-adaptations in pollinators to avoid plants using pollination by sexual swindling is unlikely unless the costs imposed by the plants to the pollinators are severe. Pollinators that become too choosy might miss mating opportunities. Here we go a step further by hypothesizing that pollinator insects could benefit from *Ophrys* flowers for both habitat matching and dispersal. Given the perfect match between the habitats of *Ophrys* and of their pollinators (e.g. Paulus 2006, Vereecken et al. 2007, Paulus, 2017, 2018), the female-like species-specific sexual pheromones emitted by *Ophrys* flowers fix pollinator males within suitable habitats where potential mates might be available for reproduction. Female hymenopterans produce pheromones that travel over large distances but the speed and directionality of wind-dispersed olfactory cues are poor. Males searching for females are thus confronted by a highly discontinuous olfactory signal of rapidly fluctuating concentration (Svensson *et al*., 2014). By increasing locally the concentration of pheromone-like molecules, *Ophrys* flowers might contribute to the location by their pollinators of suitable habitats within complex landscapes, providing insects with increased male and female reproductive success *via* increased encounter rate. By repulsing pollinators after copulation attempts, *Ophrys* flowers might favour male dispersal, potentially limiting the risk of inbreeding to which populations of haplodipoid hymenopterans are particularly exposed. We are unaware of any data supporting this hypothesis in *Ophrys*. In the Australian orchid *Drakaea glyptodon* that uses a similar pollination syndrome by sexual swindling, observations and mark-and-recapture experiments suggest that pollinators (males of the tiphiid wasp *Zaspilothynnus trilobatus*) immediately leave the area after visiting a

flower and do not visit nearby flowers within a refractory period whose duration is not mentioned (Peakall, 1990).

V. MOLECULAR BASES OF ADAPTIVE RADIATION IN *OPHRYS*

We have discussed much evidence supporting coevolutionary dynamics between *Ophrys* and their pollinators, and advocate that the adaptive radiation in *Ophrys* is promoted by intra-specific competition for mnesic pollinators. This radiation is characterized by large intra-specific variability in flower olfactory signals mimicking insect sexual pheromones. This variability is selected by the need to avoid pollinator habituation, itself selected by the ability of pollinators to recognize and memorize the identity of their mates on basis of their sexual pheromones. The key innovation that favours the adaptive radiation in *Ophrys* is the variability in female-like sexual pheromones produced by the flowers that can induce pollinator shift. Although we continuously accumulate knowledge on the eco-evolutionary drivers of this impressive adaptive radiation, the molecular bases of the striking phenotypic variation displayed by *Ophrys* spp. remain poorly understood. For example, the functional role of MADS-box (MADS stands for MCM1, AGAMOUS, DEFICIENS, SRF, four founding members of this gene family involved in controlling plant development genes that are key players in flower development in plants) has never been investigated in *Ophrys*. The relatively large genome [~ 5–10 billion base pairs in most diploid species (Leitch *et al*., 2009; Bou Dagher-Kharrat *et al*., 2013; Abreu *et al*., 2017; J. Bertrand, unpublished data; P.M. Schlüter, personal communication)] of *Ophrys* has hindered genomic investigations in this genus. As in other biological systems, this limitation is now being overcome by the increasing availability of high-throughput sequencing technologies. The genomic information available for *Ophrys* is currently limited to the three published plastid genomes [*O. iricolor*, *O. sphegodes* and *O. aveyronensis* (Roma *et al*., 2018; Bertrand *et al*., 2019)], and to the floral

transcriptomes of *O. iricolor*, *O. sphegodes*, *O. insectifera* and *O. aymoninii* (see Piñeiro Fernándes *et al*., 2019). Below, we speculate on the mechanisms that would allow phenotypic changes, particularly changes in the organic compounds composing the floral olfactory phenotypes

Candidate gene approaches have revealed the involvement of particular homologues of the stearoyl-ACP desaturases *SAD2* and *SAD5* in producing variation in organic compounds that attract different pollinators and in the resulting reproductive isolation between closely related *Ophrys* species (see Schlüter *et al*., 2011; Xu *et al*., 2012; Sedeek *et al*., 2016). In the example of *O. sphegodes* and *O. archipelagi* detailed in Section III.1*e.ii*, two mutations in the sequence coding for *SAD5* increased the attractive power of the olfactory signals produced by *O. sphegodes* (Sedeek *et al*., 2016) to its pollinator, leading to the sympatric speciation of *Ophrys sphegodes* and *O. archipelagi*. Such genetic changes in a few key genes support the idea that rapid speciation in radiating *Ophrys* is genic rather than genomic (Wu, 2001; Wu & Ting, 2004) and involves a few genes encoding traits that determine pollinator attraction. The pioneering study of Sedeek *et al*. (2014) is to date the only one to have used a genomic approach (GBS) to compare patterns of genomic differentiation of four closely related, sympatric and co-flowering *Ophrys* species (*O. archipelagi*, *O. incubacea*, *O. garganica* and O. *sphegodes*). Although they sampled a relatively small fraction of the large *Ophrys* genome, they found only a very small proportion (< 0.05%) to be strongly differentiated between pairs of species. This is in good agreement with a speciation process beginning with changes (mutations and/or gene expression modifications) occurring in a few key genes. Such changes would precede genome-wide differentiation and confirms the interest of such biological systems in allowing us to identify loci that are prominently involved at early stages of evolutionary divergence. Sedeek *et al*. (2014) highlighted new candidate genes as outlier markers of species differentiation, several of which were annotated; three of these – *Vacuolar*

Protein Sorting 45 (*VPS45*; AT1G77140.1 and *ECERIFERUM 1* (*CER1*; 1T1G02205.3) – are known to be or are putatively involved in flower odour production in plants. Although the example of *SAD2* and *SAD5* given above involves 'classical' mutations leading to amino acid change, the molecular mechanisms involved in changes in the relative concentrations of olfactory signals may rely not only on changes to DNA coding sequences, but also on up- or down-expression of genes encoding enzymes catalysing reactions leading to the assemblage of these molecules.

In spite of these examples explaining differences in *Ophrys* flower odour production, we are far from understanding the magnitude and rapidity of phenotypic variation at the scale of the genus *Ophrys*. An increasing number of studies report that the reprogramming of gene expression by insertion of transposable elements has played a key role in the adaptive evolution of plants (Lisch, 2013; Seidl & Thomma, 2017). Relevant to the *Ophrys* adaptive radiation is the finding that movement of transposable elements can cause very rapid program changes and can dramatically modify the phenotype. For example, the embedded machinery that ensures the 'copy-and-paste' mechanism of replication of long terminal repeat (LTR) retrotransposons is responsible for the expression of previously inactive colouration genes, that change the colour of fruit in some grape, orange or apple varieties (see Lisch, 2013; Zhang *et al*., 2019). Thus, transposable elements can produce variation at a rate that can vary by orders of magnitude in a few generations, and this rate can be responsive to strong environmental selection (Lisch, 2013). Therefore, transposable elements could provide a rich source of genetic and regulatory diversity among individuals, contributing to the adaptive evolution of their plant hosts to novel environments (Seidl & Thomma, 2017). Moreover, it has been shown that plants display structured genomes with transposable-element-rich regions that mediate accelerated evolution (Seidl & Thomma, 2017).

We hypothesize that genes coding for enzymes catalysing reactions leading to the organic compounds found in the odour bouquet of *Ophrys* flowers might be associated with regions where gene expression is highly variable. This could explain the huge variability observed in both biologically active and inactive organic compounds recorded in these odour bouquets. The clustering of the genes responsible for variation in odour emission in such regions would mean that reproductive isolation, and hence some speciation events, could initially be driven by purely epigenetic mechanisms. This raises the question of the strength of selection on these epigenetic mechanisms, and of phenotypic fixation by genetic assimilation. The location within regions of accelerated evolution of genes responsible for individual variation in odour bouquet would mean that speciation in *Ophrys* will depend more on changes in a few key genes than on genome-wide processes (Sedeek *et al*., 2014). Such a scenario of epigenetic control of speciation could potentially explain why the speciation rate is so high in this genus. Recent evidence that populations of cells located in the labellum of *Ophrys* flowers are polyploid (Bateman *et al*., 2018*b*) opens a new research front in the quest for the mechanisms involved in *Ophrys* speciation. In five species of *Ophrys* belonging to five different clades in the phylogeny of Breitkopf *et al*. (2015) and including basal and more derived clades, Bateman *et al*. (2018*b*) showed that the peripheral area of the labellum margin, which is rich in unicellular elongated trichomes, presented a wide spectrum of polyploid cells. Some of these trichomes were octoploid, meaning that their nuclei had undergone two cycles of endoreplication. No or weak evidence of polyploidy was found in other parts of the labellum (the speculum and the appendix, respectively). Bateman *et al*. (2018*b*) suggest that there could be overexpression of genes via the local induction of nuclear endoreplication in particular tissues. They note that overexpression of genes induced by such highly localized endoreplication could induce changes in the odour bouquet emitted by *Ophrys* flowers, and thus play a role in pollinator attraction and pollinator shift.

Hybridization–introgression among closely related species probably plays an essential role in sustaining genetic diversity, as for many other taxa in many orders. In particular, reassembly of ancient genetic variation into new combinations has been proposed as an explanation of the paradox of rapid speciation rates in radiating lineages despite slow mutation rates (Marques, Meier & Seehausen, 2019; Bertrand *et al*., 2020). As discussed in Section III.1*e.i*, hybridization is certainly one cause of speciation in *Ophrys*. Within the large number of known hybrids, it is possible to find crosses even among species belonging to the different clades identified in the phylogeny of Breitkopf *et al*. (2015). Clearly, genomic admixture can occur at many levels with the potential to create hybridogenetic species. Only a few studies have used molecular markers to investigate levels of introgression or hybridization among *Ophrys* species. The analysis of four species belonging to the recently diverging *Ophrys sphegodes* clade (according to Breitkopf *et al*., 2015) using homozygous/organellar DNA loci showed the signature of a putative hybridization event in the lineage that dominates the radiation of the clade (Cozzolino *et al*., 2020). In an attempt to assess the effect of hybridization on diversification, Cotrim *et al*. (2016) investigated the genetic structure of *Ophrys fusca* s.l. and *O. lutea* using nuclear and plastid microsatellite markers. They found that up to 12% of the genotyped plants were misclassified according to their floral phenotypes. However, these individuals did not cluster into a new group, which could indicate introgression and gene flow between the 'parent' species. Cotrim *et al*. (2016, p. 684) mention that "distinguishing hybrid introgression from common ancestry and incomplete lineage sorting remains a critical task in evolutionary studies". Admittedly, *Ophrys fusca* s.l. and *O. lutea* belong to the same clade according to a time-calibrated phylogeny of 19 species based on three nuclear genes (Joffard *et al*., 2020), two of which code for traits involved in pollinator attraction (pheromone-like molecule metabolism and flower development). This evidence of co-ancestry origin of the two study species is contrary

to the findings of Cotrim *et al*. (2016), and questions the putative hybridogenetic origin of these misclassified individuals.

More robust genetic evidence of the existence of hybridogenetic species is provided by the existence of polyploid species. Polyploidy can occur through hybridization between existing species, and induce the immediate appearance of reproductive isolation, a prerequisite for adaptive evolutionary divergence. Amich, Garcia-Barriuso & Bernados (2007) reported the presence of six polyploïd species in the Iberian Peninsula, all belonging to the section '*Pseudophrys*'. Interestingly, polyploidy within '*Pseudophrys*' appears to be restricted to the western Mediterranean basin, and may have contributed to speciation in the Iberian Peninsula, with five endemic or geographically restricted species. Polyploidy is associated with hybrid sterility and changes in gene expression between polyploid species of hybrid origin and their diploid parents. These changes occur through both gene silencing and the activation of new genes (e.g. Soltis *et al*., 2004; Schoenfelder & Fox, 2015), which might facilitate the acquisition of reproductive isolation by modifying the expression of genes coding for traits associated with the attraction of a new pollinator.

VI. NEW RESEARCH AVENUES

This investigation of the fascinating functioning of *Ophrys* was accompanied by the emergence of multiple questions (see Fig. 7 for a summary). Here, rather than a chronological enumeration of research topics that appeared successively during our review, we propose some new avenues that might provide decisive breakthroughs in the study of adaptive radiation. A first question is the relative role of epigenetics and genetics mechanisms in *Ophrys* speciation. This could be addressed by investigating the consistency of floral phenotypes over time. To what extent do floral phenotypes produced by a given individual remain stable both within and among years? Mounting evidence shows that transposable

elements can induce gene silencing or gene overexpression in plants under biotic stresses (Seidl & Thomma, 2017). It would thus be interesting to investigate how the experiences of an individual influence its phenotype over its lifetime in long-lived plants such as *Ophrys*. This question includes the consistency of their floral bouquets over time. If changes in gene expression are key in the intra-specific variation of the floral odour bouquet, we could speculate that a given individual could alter its pheromone profile both within and between years according to its current (within-year) or former (between-years) reproductive success.

We can speculate further that in the absence of pollination, the odour bouquet produced by the flowers could recruit new molecules, resulting in the formation of a brand new odour cocktail with potential to attract a new pollinator. Such changes in the absence of pollination could also holds true for other components of the phenotype but perhaps rather among years. It would also be exciting to investigate cascading effects among phenotypic traits, i.e. the extent to which changes in olfactory signals are associated with variation in other phenotype components. Even if evidence does not show changes in floral signals with time according to the previous experience of the individual plant, the latter association will be worth investigating to provide insights on the possibility that this pollination syndrome is potentially driven by epigenetic mechanisms. We suggest the adoption of an integrated framework linking molecular and cellular investigations, by considering non-linear and interactive relationships (including feedbacks) among all 'omic' compartments (Baguette, Legrand & Stevens, 2015) and by comparing the expression of genes coding for traits responsible for pollination attraction over time in cells from vegetal (e.g. leaves) and reproductive (flowers) tissues.

We also recommend the construction of an exhaustive and reliable molecular phylogeny of the genus *Ophrys*. The currently most useful phylogeny (Breitkopf *et al*., 2015) is based on less than 10% of the currently known species. Cusimano & Renner (2010) showed

that at least 80% of species should be included in a phylogenetic analysis to provide sound estimates of diversification rates. Due to the low differentiation of the *Ophrys* genome, such a phylogeny should be based on the use of an appropriate number of loci, each probably containing only a limited amount of information. These loci should include flanking regions that would be conserved enough among *Ophrys* species to be amplified, but should be also as variable as possible to detect differentiation among clades and species [see Loiseau *et al*. (2019) for a recent example of the use of baits protocol, which consists in the simulateous targeted capture and sequencing of hundreds to thousands of nuclear genes]. Besides providing a definitive response to the endless controversy about species definition in *Ophrys*, this phylogeny could provide insights into the genetic material available for potential speciation events based on epigenetic mechanisms, if they are involved. Accordingly, a phylogeny based on DNA sequences could be compared to phenotypic data to disentangle the relative roles of phylogenetic constraints and of the evolution of floral phenotypes [Byers, Xu & Schlüter (2017) proposed an integrative approach linking the molecular mechanisms of adaptation and speciation, and Joffard *et al*. (2020) provide a seminal analysis of this topic]. The availability of an exhaustive and reliable molecular phylogeny based on DNA sequences would also provide insights into the history of this radiation, which is of particular interest in attempts to detect rapidly evolving clades. Using detailed genomic data on the Orchidaceae that are now available to calibrate the radiation in *Ophrys*, it would be possible to infer more precisely the role of environmental changes in the formation of different clades and their relative speciation rates.

In addition, a molecular phylogeny based on DNA sequences would allow comparisons of the distribution range and metapopulation dynamics of parent, ancient and derived, recent taxa. This will allow a comparison of data collected across different scales of biological organization, from individual features to species distribution ranges, through to

population and metapopulation dynamics. Accordingly, the reproductive success of individuals belonging to parent and derived species could be monitored over time and related to individual genotypes for traits involved in pollinator attraction and phenotypic variability. Reproductive success could be related to population features, such as nearest-neighbour distance, pollinator abundance, conspecific density, the presence and density of congeneric species, and habitat shape and area. Such data should be collected in several metapopulations over time, to generate an integrated data set linking individual fitness to population and metapopulation dynamics in spatially, phenotypically and genetically explicit contexts, and compared to predictions of ecological niche models and to the real distribution of the respective species. This procedure should provide first-hand data on the speed of expansion of new species and insights on the advantages of the colonization of competitor-free space.

It will be possible to integrate these data into the framework of adaptive radiation introduced by Donoghue & Sanderson (2015), who advocated that the 'key innovation' hypothesis should be nuanced toward more multi-causal explanations assembled step-wise through a tree. They introduced the term 'synnovation' (a hybrid of 'synergy' and 'innovation') for an interacting combination of traits with a particular consequence ('key synnovation' in the case of increased diversification rate), and the term 'confluence' for the sequential coming together of a set of traits (innovations and synnovations), environmental changes, and geographic movements along the branches of a phylogenetic tree. Here we consider that pollination by sexual swindling is the key innovation leading to the radiation of *Ophrys*, and we anticipate that pollinator shifts, especially those leading to the attraction of new bee genera, might be synnovations. These synnovations would rely on changes in the composition of pseudo-pheromone bouquets and on flower morphology, and would thus concern a few changes in gene sequences and/or expression. However, we advocate that a strict application of this framework to the adaptive radiation of *Ophrys* is preliminary at this

stage of knowledge of speciation within this genus (Fig. 7). For example, the identification of this basal clade of the genus still remains controversial, currently precluding any conclusive statement about synnovation and confluence. Indeed, the basic data leading to the identification of confluences are currently missing.

Finally, combined with similar data on pollinators, such comparisons will provide detailed insights into the best way to conserve the evolutionary potential of this genus in the current context of anthropogenic global change. We particularly encourage collaborative work using similar approaches and protocols among the different scientific teams working in the Mediterranean region.

A third issue concerns coevolution between plants and their pollinators. We proposed that pollinators could benefit from the presence of *Ophrys* for both habitat matching and dispersal. To the best of our knowledge, there are no conclusive data to support this hypothesis. This might be addressed experimentally, by recording in closed arenas the behaviour of pollinators presented with receptive females or *Ophrys* flowers. Observations of the males could inform on the respective effects of females and flowers on the propensity of the male to remain in the arena or to leave, corresponding to habitat-matching and dispersal, respectively. Similar experiments would allow to compare the consistency of pollinator behaviour during copulation with their legitimate females and during copulation attempts with flowers. Hymenopteran males that display similar behaviour in both treatments show no indication that they can detect flower lures, and hence flowers have managed to achieve complete mimicry; if the post-mating behaviour of males differs between the treatments, we should expect that they associated the copulation with a flower with some costs. It would be interesting to perform such experiments with both parent and derivate plants as inferred from a dated molecular phylogeny, to investigate to what extent the plant–pollinator relationship has been reinforced over time since a pollinator shift led to speciation.

VII. CONCLUSIONS

(1) Bee orchids (*Ophrys* spp.) use pollination by sexual swindling: their flowers mimic signals emitted by receptive females of pollinating insects (mainly solitary bees). This highly specialized pollination strategy has led to the evolution of striking morphological similarities between flowers of bee orchids and the females of their species-specific pollinators.

(2) The fundamental role of olfactory signals in the functioning of pollination by sexual swindling triggered a profusion of studies disentangling the relationships between *Ophrys* flowers and their pollinators. Combined field and laboratory studies identified similarities between olfactory signals in derived *Ophrys* species sharing the same pollinator species, and differences between closely related *Ophrys* species with different pollinator species. Resolving the phylogeny of *Ophrys* will be key to being able to identify (groups) of related species and to considering their evolutionary relationships with their pollinators. However, a consensual *Ophrys* phylogeny is still lacking with even the definition of a 'species' of bee orchid vigorously debated. Two main species concepts are in conflict: a definition based on DNA sequence homologies, and a definition based on prezygotic isolation by attraction of species-specific pollinators. According to the first definition, there are currently around ten species of bee orchids, whereas adoption of the prezygotic isolation criterion leads to the recognition of several hundred species.

(3) The unified species definition validates the prezygotic isolation criterion that asserts the existence of several hundred *Ophrys* species. Given the recent origin of this genus (4.9 million years), this explosion of species over such a short time scale corresponds to a radiation. We have tried to highlight the processes by which this radiation occurred, and to what extent it is adaptive. We found that one current hypothesis to explain speciation in *Ophrys*, i.e. negative frequency-dependent selection does not explain the high intra-specific

variability present in the olfactory signals used by *Ophrys* flowers to attract species-specific pollinators. Instead, we found a battery of independent indications showing that the high speciation rate in *Ophrys* is a result of intra-specific competition between plants competing for pollinators that have high cognition and memory abilities. Hymenopteran pollinators developed individual mate recognition both to avoid mating with kin and to avoid wasting time and energy in courting previously mated females. Flowers of *Ophrys* plants must thus differ from each other to be attractive to their mnesic pollinators. Accordingly, intra-specific competition could have generated the large number of distinct olfactory phenotypes seen in field and laboratory observations, but which disagree with the predictions of the negative frequency-dependent selection hypothesis that predicts the existence of only a few phenotypes.

(4) Variation of olfactory phenotypes could by chance drive random crossings of peaks in the olfactory selective landscape of the pollinator guild that is syntopic to each particular *Ophrys* population and thus attract a new pollinator species. This event could lead to the prezygotic isolation of a new taxon if it occurred simultaneously in several plants within the same population, followed by directional selection on flower morphology to reinforce the attraction of the new pollinator.

(5) The sexual-swindling pollination strategy of bee-orchids may be considered a key innovation triggering an adaptive radiation by intra-specific competition for mnesic pollinators. This radiation is adaptive because the newly isolated *Ophrys* taxa benefit from competitor-free space. This finding reshapes and unifies the voluminous scientific literature dealing with *Ophrys* into a common and integrated eco-evolutionary framework, and generates several exciting suggestions for research avenues, including (1) the molecular mechanisms involved in adaptive radiations; (2) the relative roles of sympatric and allopatric

speciation in adaptive radiations; and (3) the asymmetric plant–pollinator coevolution that may underlie this particular adaptive radiation.

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X. SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

Table S1. Families of pollinators of Ophrys species from the two rapidly diverging clades identified by Breitkopf *et al*. (2015).

Table 1. Spatial and temporal variation of reproductive success (RS) in three *Ophrys* species assessed as the proportion of the number of pollinated flowers/total number of flowers, using a standardized protocol (Vandewoestijne *et al*., 2009). *N* = number of populations.

Species	**Inter-site range of RS**	**Inter-annual range of RS**
Ophrys fuciflora	0–19.4%, *N* = 4	3.7–19.4%, *N* = 4
Ophrys insectifera	2.4–40.7%, *N* = 7	23.4–40.7%, *N* = 5
Ophrys sphegodes	3.3–51.7%, *N* = 4	38.3– 51.7%,, *N* = 2

Table 2. Proportions of different families of pollinators within the two rapidly diverging *Ophrys* clades identified by Breitkopf *et al*. (2015). The number of species in each clade is in parentheses. *Ophrys* systematics mainly following Delforge (2016); pollinator data mainly from Paulus (2018). See online Supporting information, Table S1 for detailed data.

	Pollinators					
Clade	Andrenidae	Apidae	Colletidae	Megachilidae	Scarabeidae	Sphecidae
O. fusca (*N* = 73)	0.84	0.08	0.04	0.01	0.03	0
O. sphegodes (*N* = 78)	0.44	0.27	0.09	0.19	0	0.01

Table 3. Proportion of potted flowers that received copulation attempts by pollinator males in the field. Number of tested flowers is in parentheses (data from Stökl *et al*., 2008).

Species	***Andrena nigroaenea* alone**	***Andrena morio* alone**	**Both *Andrena* species**
Ophrys lupercalis (N = 10)	0.80	0	0.20
Ophrys eleonorae (*N* = 9)	0.22	0.56	0.22
Hybrids (*N* = 14)	0.36	0.36	0.28

Table 4. Pollinator identity and body size of the three species of the *O. insectifera* clade. Data from various sources compiled in Triponez *et al*. (2013).

Orchid species	**Main pollinator species**	**Pollinator body length (mm)**
Ophrys insectifera	*Argogorytes mystaceus* (and *A. fargei*) (Hymenoptera: Crabronidae)	9.5-12
Ophrys subinsectifera	*Sterictophora gastrica* (Hymenoptera: Argidae)	6.8-7.2
Ophrys aymoninii	*Andrena combinata* (Hymenoptera: Andrenidae)	8.5-9.5

Table 5. Flowering periods (months of the year) of species of the *Ophrys attaviria* group from Crete and their *Andrena* pollinators. The early-flowering species are isolated from the latest three because of the absence of overlap in their flowering period. Data from various sources including Delforge (2016) and Paulus (2018).

		Flowering period				
Ophrys	***Andrena***	**I**	**II**	**III**	**IV**	**V**
O. siatica	*A. nigroaenea*					
O. creticola	*A. bimaculata*					
O. thriptiensis	*A. bicolor*					
O. cinereophila	*A. cinereophila*					
O. cressa	*A. merula*					
O. creberrima	*A. creberrima*					
O. pallidula	*A. combinata*					
O. kedra	*A. variabilis*					

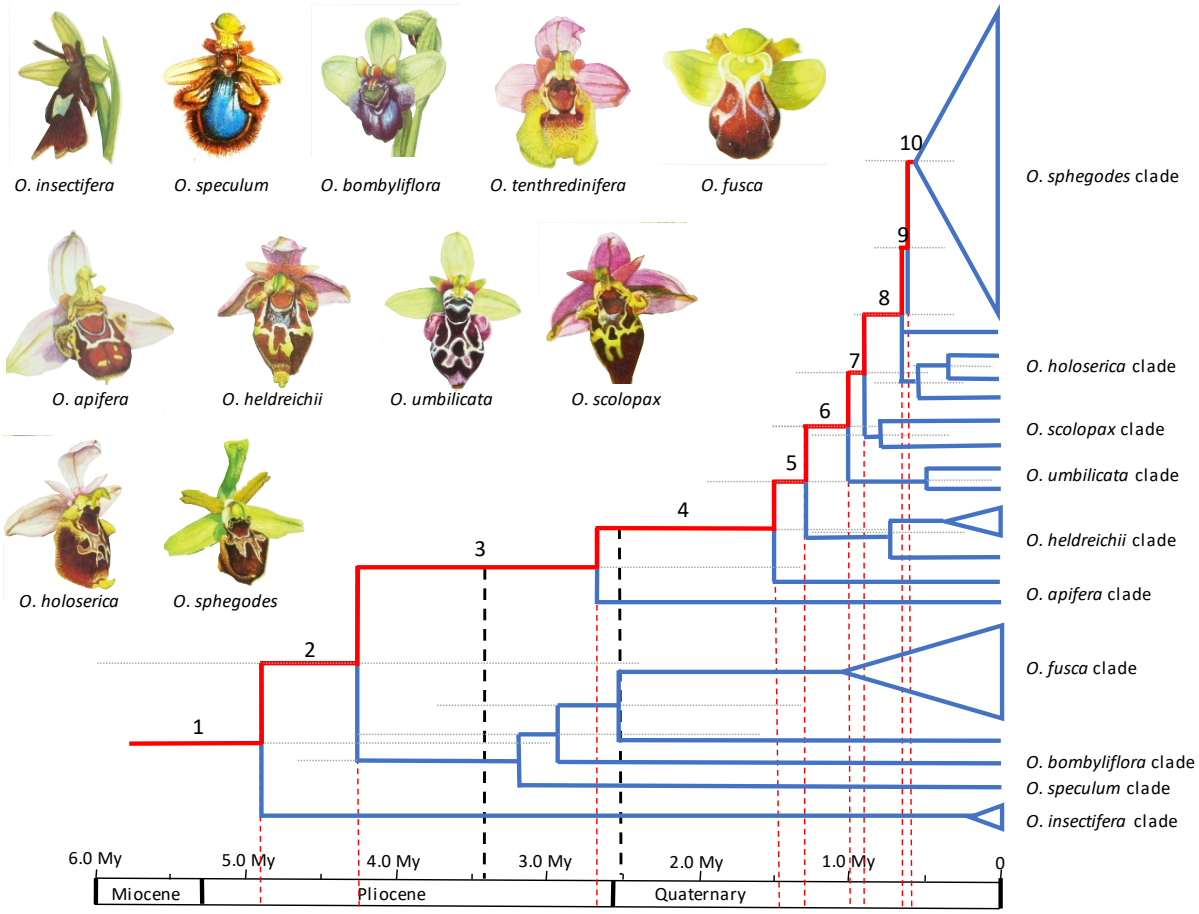

Fig. 1. Extreme adaptive radiation in bee orchids (*Ophrys* spp.). In this group, several hundred species evolved around the Mediterranean basin over less than 4.9 million years (My), a diversification rate almost unrivaled worldwide. Time-calibrated (in My) phylogenetic relationships between 11 clades inferred from DNA sequences from 37 species and their floral phenotypes (red dotted lines). Triangles depict rapid ongoing radiations; the diversification rate in these clades is higher than the mean diversification rate for the genus (modified from Breitkopf *et al.*, 2015). Interestingly, there are two phases of diversification (black dotted lines): there are only three clades up to 3.4 My ago and seven clades up to 2.5 My ago, after which a more rapid diversification gave rise to the present 11 clades. In agreement with other analyses, this molecular phylogeny confirms the monophyly of the

genus *Ophrys*, and invalidates its division into two sub-genera grouping species with either an abdominal (*Pseudophrys*, basal) or cephalic (*Euophrys* [invalid name], derived) position of the pollinator on the plant during copulation attempts. *Ophrys* paintings from Landwehr (1977).

Fig. 2. Flower of *Ophrys bombyliflora* with its stigmatic cavity filled by pollinia. Picture courtesy of Jean Claessens.

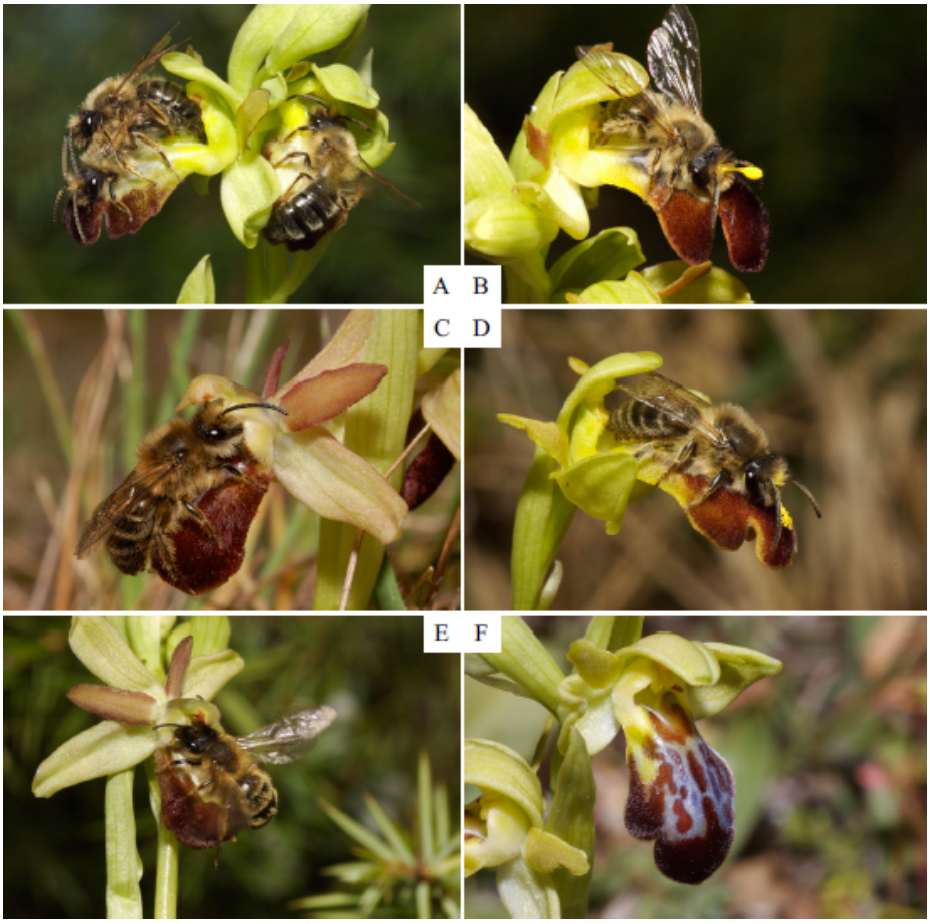

Fig. 3. Copulation attempts of (A) males of *Andrena nigroaenea* (Hymenoptera: Andrenidae) in both the 'abdominal' and the 'cephalic' positions on the flower labella of *Ophrys lupercalis*; (B) male of *A. nigroaenea* in the 'abdominal' position on a flower of *O. lupercalis* with pollinaria on its head; (C) male of *Colletes cunicularius* (Hymenoptera: Colletidae) in the 'cephalic' position on the flower labellum of *O. arachnitiformis*; (D) male of *C. cunicularius* on the flower labellum of *O. lupercalis* with pollinaria on its head; (E) male of *A. nigroaenea* in 'cephalic' position on the flower labellum of *O. arachnitiformis*. (F) A flower of the natural hybrid between *O. arachnitiformis* and *O. lupercalis*. All photographs by N.J. Vereecken. Reproduced from Vereecken *et al*. (2010).

Fig. 4. Match between an *Ophrys speculum* flower and the female of her pollinator, the scolid wasp *Dasyscolia ciliata ciliata* (Hymenoptera: Scoliidae). The glossy blue speculum on the labellum of the flower corresponds to the bluish wings of the pollinator's female, the lateral lobes of the labellum mimic her legs and the brown hairs bordering the labellum are similar to her pilosity. Picture reprinted from Paulus (2006), with permission.

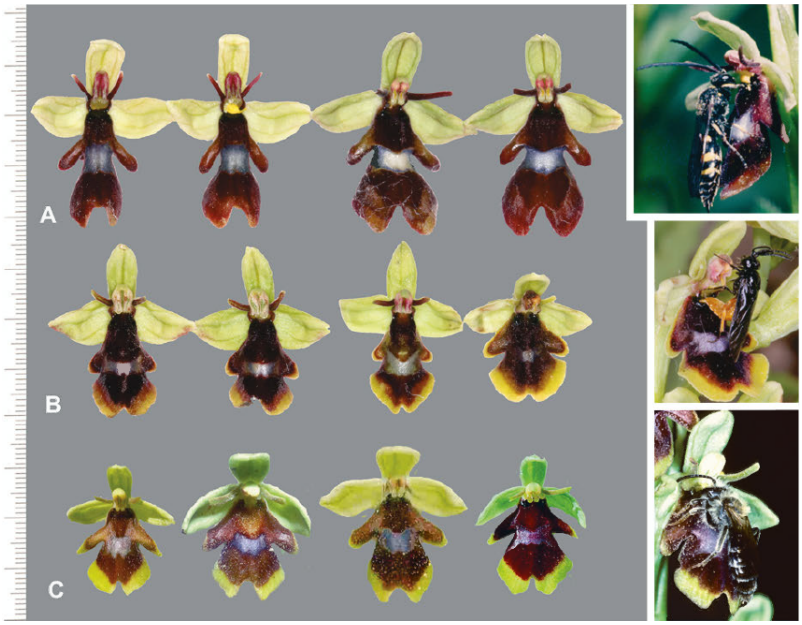

Fig. 5. Flowers of the three species of the *Ophrys insectifera* clade and their pollinators. (A) *Ophrys insectifera* and *Argogorytes mystaceus*. (B) *Ophrys subinsectfera* and *Sterictophora gastrica*. (C) *Ophrys aymoninii* and *Andrena combinata*. Picture reprinted from Paulus (2017), with permission.

Speciation in the *Ophrys insectifera* clade

Eco-evolutionary processes

Variation in olfactory signals
- *O. insectifera:* larger proportions of alkenes, alkadienes
- *O. aymoninii:* three specific fatty acid esters
- *O. subinsectifera:* three specific aldehydes

Inter-individual variation in
- labellum length
- number of flowers
- distance between flowers

O. insectifera/ O. aymoninii
O. insectifera/ O. subinsectifera

Environmental processes

Differences in habitat selection
- elevation
- slope
- geometry

O. insectifera/ O. aymoninii
No data for O. subinsectifera

Differences in pollinator biology
To be explored

Low genomic differentiation
Signals of introgression

Key innovation: olfactory signals
Target of directional selection: floral morphology (labellum, pollinia)

Fig. 6. Current knowledge about potential drivers of speciation in the *Ophrys insectifera* clade (photo © S. Witzthum) [based on Triponez *et al*. (2013) and Joffard *et al*. (2016)]. Speciation in the *Ophrys insectifera* clade is likely to have involved a combination of eco-evolutionary processes (inter-individual variation in olfactory signals, flower shape, flower number and distance between flowers) and environmental processes (differences in habitat selection and pollinator biology). That speciation is recent is revealed by low genomic differentiation and signals of introgression among *O. insectifera* and the two newer species. We suggest that a change in olfactory signals is key in speciation, followed by directional selection of flower morphology.

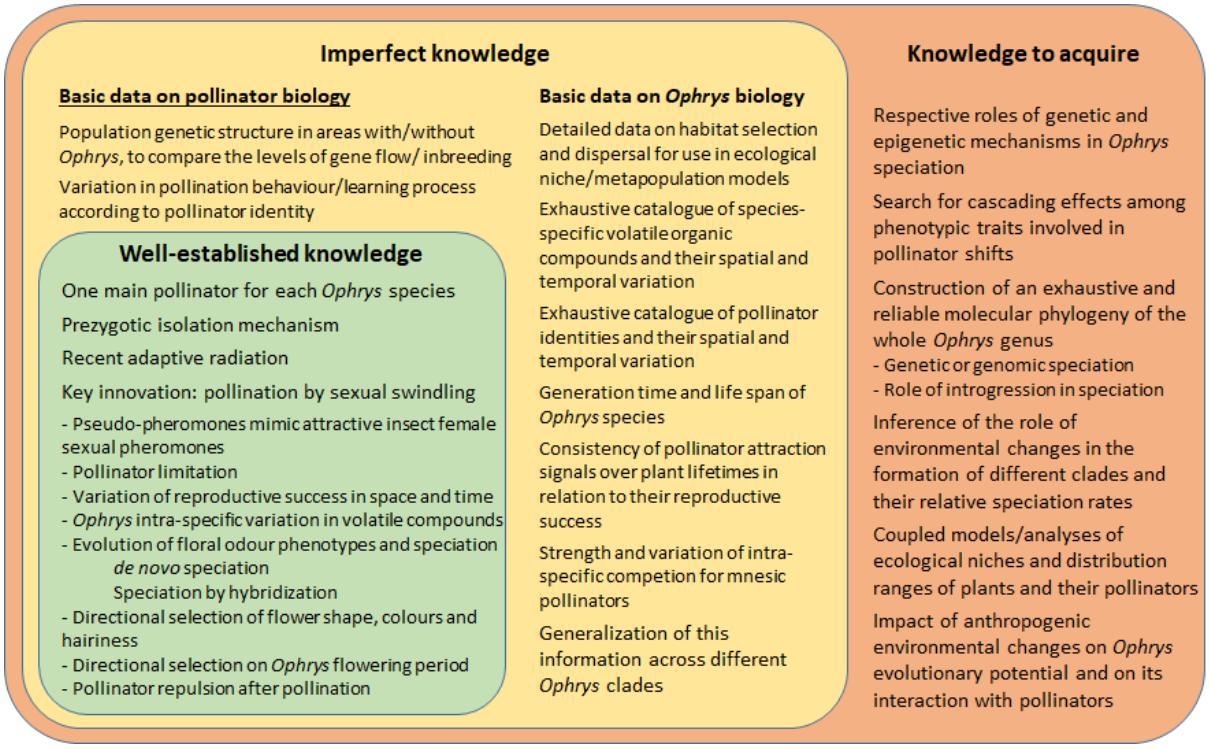

Fig. 7. General overview of the state of knowledge about *Ophrys* speciation, with emerging research questions.