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Pollinator convergence and the nature of species' boundaries in sympatric Sardinian *Ophrys* (Orchidaceae)

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• *Background and Aims* In the sexually deceptive *Ophrys* genus, species isolation is generally considered ethological and occurs via different, specific pollinators, but there are cases in which *Ophrys* species can share a common pollinator and differ in pollen placement on the body of the insect. In that condition, species are expected to be reproductively isolated through a pre-mating mechanical barrier. Here, the relative contribution of pre- vs. post-mating barriers to gene flow among two *Ophrys* species that share a common pollinator and can occur in sympatry is studied.
• *Methods* A natural hybrid zone on Sardinia between *O. iricolor* and *O. incubacea*, sharing *Andrena morio* as pollinator, was investigated by analysing floral traits involved in pollinator attraction as odour extracts both for non-active and active compounds and for labellum morphology. The genetic architecture of the hybrid zone was also estimated with amplified fragment length polymorphism (AFLP) markers, and pollination fitness and seed set of both parental species and their hybrids in the sympatric zone were estimated by controlled crosses.
• *Key Results* Although hybrids were intermediate between parental species in labellum morphology and non-active odour compounds, both parental species and hybrids produced a similar odour bouquet for active compounds. However, hybrids produced significantly lower fruit and seed set than parental species, and the genetic architecture of the hybrid zone suggests that they were mostly first-generation hybrids.
• *Conclusions* The two parental species hybridize in sympatry as a consequence of pollinator overlap and weak mechanical isolation, but post-zygotic barriers reduce hybrid frequency and fitness, and prevent extensive introgression. These results highlight a significant contribution of late post-mating barriers, such as chromosomal divergence, for maintaining reproductive isolation, in an orchid group for which pre-mating barriers are often considered predominant.

Key words: AFLP markers, floral scent variation, hybrid zone, hybrid fitness, *Ophrys iricolor*, *Ophrys incubacea*, reproductive isolation, sexual deception.

INTRODUCTION

Evolution of reproductive barriers, often grouped into pre- and post-mating isolating mechanisms, is of central importance for speciation (Coyne and Orr, 2004). Typically, maintenance of species boundaries is the result of the combination of both mechanisms, but the relative contribution of pre- and post-mating barriers is highly variable among plant groups and depends on peculiarities of their reproductive biology (Grant, 1971; Schemske, 2000). Pre-mating barriers have received considerable attention in plant lineages characterized by a high degree of floral diversification, such as orchids, which led to the assumption that pollinator specificity of orchid–insect interactions is of primary importance in maintenance of species boundaries in this group of flowering plants (Van der Pijl and Dodson, 1966; Dodson and Gillespie, 1967; Gill, 1989; Tremblay *et al.*, 2005). Consequently, the role of post-mating barriers has rarely been investigated in studies of

reproductive isolation and speciation in orchids (Cozzolino *et al.*, 2005; Scopece *et al.*, 2007, 2008). Recent studies have provided evidence for the prevalent role of post-mating barriers in orchid species with lower pollinator specialization such as food-deceptive orchids (Moccia *et al.*, 2007; Scopece *et al.*, 2008), whereas pre-mating barriers (i.e. pollinator specificity) were shown to be much more decisive in the reproductive isolation of Mediterranean sexually deceptive orchids (Scopece *et al.*, 2007). Although Scopece *et al.* (2007) have recently reported one of the first studies of post-mating isolation in sexually deceptive *Ophrys* species, the relative contribution of pre- vs. post-mating barriers to reproductive isolation in these plants remains relatively unknown (Cozzolino and Widmer, 2005) as it is in the corresponding sexually deceptive Australian orchids (Bower, 1996).

The Mediterranean genus *Ophrys* is remarkable for its highly complex and variable floral morphology. *Ophrys* flowers mimic visual, tactile and olfactory cues of females of their pollinating species (reviewed by Schiestl, 2005). Cross-pollination in these orchids is brought about by a process termed pseudocopulation:

male pollinators respond to these 'false females' (i.e. the orchid flowers) by attempting to copulate with the orchid labellum, thereby removing or delivering pollen (Kullenberg, 1961). As in sexually deceptive orchids more generally (Schiestl, 2005), the most important signal employed by *Ophrys* flowers for pollinator attraction is chemical mimicry of the sex pheromone of the virgin female (Schiestl *et al*, 1999; Schiestl, 2005; Stökl *et al.*, 2007). Sex pheromone-mimicking compounds emitted by *Ophrys* flowers represent complex blends of ubiquitous odour compounds, mostly long-chain alkanes and their derivatives in which in the majority of cases the specific ratio of alkanes and alkenes of different chain lengths provides the basis of specific pollinator attraction (Schiestl *et al*, 1999; Schiestl and Ayasse, 2002; Ayasse *et al.*, 2003; Mant *et al.*, 2005*a*; Stökl *et al.*, 2005, 2007).

However, in some cases, morphologically distinct sympatric *Ophrys* species can attract the same pollinator. It is known that, at least in part, reproductive isolation is maintained by mechanical isolation due to differing placement of pollen on pollinators (Kullenberg, 1961; Borg-Karlson, 1990). The difference in pollen placement between orchid species is promoted by contrasting presentation of the hairs on the labellum that encourage either a forward or reverse orientation of the pollinator such that pollen is deposited on either the head or the abdomen, respectively (Ågren *et al.*, 1984; Pirstinger, 1996). Such differences in labellum micromorphology characterize two sections within *Ophrys*: species in sect. *Ophrys* deliver pollinia on the head of their pollinator, whereas those in sect. *Pseudophrys* place pollinia on the abdomen (Ågren *et al.*, 1984; Paulus and Gack, 1990). Despite low interspecific taxonomic resolution obtained from recent molecular studies, several phylogenetic reconstructions based on plastid and nuclear ribosomal markers independently showed that sect. *Pseudophrys* is monophyletic (Soliva *et al.*, 2001; Bateman *et al.*, 2003; Devey *et al.*, 2007). Consequently, the hypothesis holds that two *Ophrys* species from different sections (i.e. *Ophrys* vs. *Pseudophrys*) that attract the same pollinator by producing the same scent bouquet can co-occur in sympatry, share a pollinator species and remain reproductively isolated through a pre-mating (here, mechanical) barrier. Yet, despite the apparent strength of pre-mating isolation mechanisms, the formation of hybrids among *Ophrys* species with different placement of pollinia suggests that this mechanical barrier is not absolute and allows for some degree of hybridization in zones where species with the same pollinator overlap (Stöckl *et al.*, 2008). However, since the role of post-mating barriers has been largely overlooked in studies of *Ophrys* pollination, the phenomenon of natural hybridization in this group of orchids provides a unique opportunity to investigate the components of reproductive isolation by testing the relative contribution of pre-mating (ethological and mechanical isolation) vs. post-mating barriers in the maintenance of species integrity between sympatric species and in the evolution of hybrid zones (Martinsen *et al.*, 2001; Lexer *et al.*, 2005).

In this study, a natural hybrid zone between two *Ophrys* species, *O. iricolor* and *O. incubacea*, that share the same pollinator was investigated. Specifically, a combination of methodological approaches was used to characterize differences in morphological and chemical floral characters among taxa, status of intermediate phenotypes, genetic architecture of the hybrid zone and fitness of hybrids under natural conditions to estimate the relative contribution of pre- vs. post-mating barriers to gene flow among species.

MATERIALS AND METHODS

Orchid species profiles and study site

Like many European terrestrial orchids, *Ophrys iricolor* (sect. *Pseudophrys*) and *O. incubacea* (sect. *Ophrys*) grow in calcareous grasslands, garrigues and open woodlands. Both species have a Mediterranean distribution, *O. incubacea sensu lato* being distributed from Spain to Italy and Albania, and *O. iricolor* in Corsica and Sardinia but also known to occur in Tunisia and Algeria. The two orchids bloom at the same period of the year and attract the same pollinator species, patrolling males of *Andrena morio* (Hymenoptera, Andrenidae; Delforge, 2005; Stökl *et al.*, 2007). Despite the lack of behavioural isolation, a mechanical barrier usually reproductively isolates *O. iricolor* from *O. incubacea* through the different position of the pollinators during pseudocopulation, resulting in deposition of pollen masses (pollinia) on different parts of the pollinator. Typically, males of *A. morio* perform pseudocopulation in an abdominal position on the flower labellum of *O. iricolor* and withdraw pollinia on the tip of their abdomen, whereas pseudocopulation is performed in a cephalic position in *O. incubacea*, resulting in the deposition of the pollinia on the head of the insect. The two species have been found growing in sympatry in many localities, and they occasionally hybridize in a natural population in southern Sardinia (Cagliari, Italy; Scrugli and Manca Mura, 1996). When in contact on Sardinia and Corsica islands, hybridization has been reported, and hybrids have previously been described on morphological bases as *O.* × *tavignanensis* (Mathé *et al.*, 1997).

Sampling of floral odours

Individual labella of fresh, unpollinated flowers of each taxon (*O. iricolor* $n = 25$; *O. incubacea* $n = 28$; hybrids $n = 18$) were extracted in 200 μL of hexane for 1 min, and extracts were then stored at −20 °C prior to chemical analyses. A 100 ng aliquot of *n*-octadecane (C18) was added to each sample as internal standard, and 1 μL aliquots of the extracts was injected splitless at 50 °C (1 min) into an Agilent 6890 gas chromatograph (GC), followed by opening of the split valve and programming to 300 °C at a rate of 10 °C min^{-1}. The GC was equipped with a HP-5 column [30 m × 0·32 mm (diameter) × 0·25 mm (film thickness)] and a flame ionization detector (FID); helium was used as the carrier gas. All odour compounds were classified by retention time, their absolute amounts were calculated by the internal standard method described by Mant *et al.* (2005*a*) and the relative proportions (%) were calculated.

DNA extraction and molecular analyses

Plant material from fresh flowering plants of each orchid taxon (*O. iricolor* $n = 30$; *O. incubacea* $n = 25$; hybrids $n = 34$) from the same data set used for floral odour analyses was sampled in

the spring of 2005 to conduct molecular analyses. The DNA extraction method consists of a slight modification of the 2× cetyltrimethyl ammonium bromide (CTAB) protocol of Doyle and Doyle (1987). The material was macerated in 700 µL of standard CTAB buffer, incubated at 60 °C for 30 min, extracted twice with chloroform–isoamyl alcohol, precipitated with iso-propanol and washed with 70 % ethanol. DNA was re-suspended in 50 µL of water. To distinguish between different hybrid classes, such as first-generation hybrids (F_1) or early back-crosses, the amplified fragment length polymorphism (AFLP) method was used. These genetic markers offer several advan-tages over morphological characters including simple modes of inheritance, independence among markers and access to a far larger set of genetic characters for identifying and characterizing hybrids. They therefore represent a useful technique to detect even low levels of introgression (Mueller and Wolfenbarger, 1999). AFLP analyses were performed using a modified version of Vos *et al.* (1995). Restriction digestion was conducted using restriction enzymes *Eco*RI and *Mse*I on 300 ng of genomic DNA. Ligation of *Eco*RI and *Mse*I adaptors to restriction frag-ments took place concurrently with restriction digestion. A pre-amplification PCR of the restriction fragments was conducted using a template of 2 µL of restriction–ligation product. Primers for pre-amplification were *Eco*RI and *Mse*I primers with one additional selective nucleotide. A second selective amplification was conducted with 2·5 µL of a 1:20 dilution of the pre-amplification product. The primers used were the same as for pre-amplification, but with one or two additional selective nucleotides. After an initial screening, six primer pairs (AGC–CGG, AGC–CCA, ATG–CGG, AGC–ACTG, ACC–ACTG and ACC–ACAC) were used. Fragment separation and detection were performed on an ABI 3100 AVANT DNA sequencer. Fragment sizes (in bp) were determined with Genotyper 3·7 soft-ware, using an internal size standard (GeneScan Rox500, Applied Biosystems).

Fitness estimates in situ *and post-mating isolation experiments*

Ninety-eight individuals in the hybrid zone, representing each orchid taxon (*O. iricolor* $n = 41$; *O. incubacea* $n = 38$; hybrids $n = 19$), were labelled individually, and their repro-ductive success was assessed by counting (*a*) the number of fruits produced over the numbers of flowers per inflorescence and (*b*) the proportion of viable seeds produced per fruit.

To highlight the presence of embryos, seeds from each fruit were then coloured by immersion in a 50 % solution of lactic acid overnight (see, for example, Cafasso *et al.*, 2005). Coloured seeds were subsequently observed under an optical microscope (×100 magnification), and seeds were assigned to two categories (viable vs. non-viable seeds) depending on the presence of embryos. Samples of 300 seeds per fruit were scored to estimate the percentage of viable seeds for each fruit. Ultimately, the overall seed set was assessed by counting the number of viable vs. non-viable seeds (Ellis and Johnson, 1999).

Hand-pollination experiments were performed in 2007 in a common garden design on plants collected in 2005 to measure post-mating isolation indices. To prevent uncontrolled pollination, the plants were placed in a cage covered with a thin net before anthesis. Pollination experiments were

performed as described in Scopece *et al.* (2007). All crosses were performed bi-directionally.

Flower micromorphology

Ten individual flowers of each taxon (their species or hybrid status confirmed by molecular analysis) were sampled and pre-served in water:ethanol:glycerol (50 : 42 : 8 v/v/v). The individ-ual flowers were then dehydrated in a graded ethanol series and critical-point dried in liquid CO_2. All samples were mounted on aluminium stubs and coated to approx. 30 nm with gold. Specimens were observed under an FEI Quantas 200 ESEM.

Statistical analyses

Floral odour differentiation among taxa was investigated by performing canonical discriminant function (CDF) analyses using (*a*) the biologically active compounds identified by Stökl *et al.* (2007) and (*b*) the non-active compounds recorded in floral odour extracts. The partitioning of odour variance among vs. within taxa was investigated by adapting the analy-sis of molecular variance (AMOVA) framework for analysis of odour (Mant *et al.*, 2005*b*). A pairwise individual-by-individual Euclidean distance matrix calculated from the rela-tive amounts of odour compounds in SPSS 13·0 (SPSS Inc., Chicago, IL) was used as input file. This distance matrix was then transferred to GenAlEx 6 (Peakall and Smouse, 2005) for analysis. Random permutations ($n = 999$) were used to test for significant differences in odour partitioning among and within taxa. To reduce the number of variables for CDF to fewer than the number of samples, a preliminary principal component analysis (PCA) was carried out with all 36 compounds to reduce the number of variables for analysis of the differentiation in patterns of non-active compounds among orchid taxa. All principal components generated by this PCA were then used to perform the CDF analysis.

The AFLP profiles were scored in terms of presence of each variable marker in each individual, and a binary data matrix was constructed. The matrix was used to calculate an individual pairwise genetic distance matrix by employing the software package GenAlEx V5 (Peakall and Smouse, 2005), and a princi-pal co-ordinate analysis (PCO) and AMOVA were carried out based on the genetic distances.

The genetic marker data were then used to calculate a mol-ecular hybrid index for each individual. The hybrid index is an estimate of the proportion of alleles inherited from one of two parental species (Rieseberg and Carney, 1998). For the treat-ment here, one species was designated as the reference species and the other as the alternative species. The hybrid index ranges between zero and one, corresponding to pure individuals of the alternative and reference species, respect-ively (Buerkle, 2005). For identifying hybrid categories, the software HINDEX, which applies a maximum-likelihood approach for estimation of the hybrid index was used (Buerkle, 2005). Additionally, a marker would be considered as species-specific if it occurred in 100 % of the individuals in one parental species but was absent from the other.

Reproductive success (i.e. fruit set and proportion of viable seeds) was compared among taxa by a two-independent-samples test procedure (Mann–Whitney

U-test). Pairwise tests were used to define which taxa had significantly different values. All statistical analyses were performed using SPSS 13·0.

RESULTS

Floral odour differentiation among taxa

Our analyses of floral odour of *O. incubacea*, *O. iricolor* and their hybrids revealed the same set of 69 peaks in all samples, including the 36 odour compounds found to be active in males of *A. morio* by Stökl *et al.* (2007). These biologically active compounds (BACs) consist primarily of long, straight-chain cuticular hydrocarbons from 21 to 31 carbon atoms and their derivatives, such as alkenes (monounsaturated alkanes) with the double bond at positions 5, 7 and 9, and alkadiene C29 (Stökl *et al.*, 2007).

Overall, the multivariate analyses of floral odours showed weaker differentiation among taxa in BACs than in non-active compounds (Fig. 1A active compounds, high Wilks' λ values: $W\lambda_1 = 0.492$; $W\lambda_2 = 0.961$, associated $P_1 < 0.001$ and $P_2 = 0.627$; low canonical correlations: $cc_1 = 0.699$ and $cc_2 = 0.196$; Fig. 1B non-active compounds, low Wilks' λ values: $W\lambda_1 = 0.031$; $W\lambda_2 = 0.223$, associated P_1 and $P_2 < 0.001$; high canonical correlations: $cc_1 = 0.927$ and $cc_2 = 0.882$). The CDF analysis performed with all BACs showed a considerable overlap among samples and taxa (Fig. 1A; 92 % of total odour variance lies within taxa, $P < 0.01$). In particular, the analysis showed a greater overlap in floral odour between the two parents, the hybrids forming a relatively separate cluster (Fig. 1A; only 53·5 % of the cross-validated samples were correctly classified). The CDF analysis performed with the non-active compounds yielded a different pattern with virtually no overlap in the proportions of these compounds among parental species and hybrids (Fig. 1B; 84·5 % of the cross-validated samples were correctly assigned to their taxa). However, the three taxa form a relatively compact cluster in olfactory space (90 % of the total odour variance lies within taxa, $P < 0.01$).

Genetic affinities among taxa in the hybrid zone

The AFLP analysis produced a total of 253 polymorphic markers. The hybrid index analysis based on all markers revealed that the hybrid zone consisted mainly of F_1 generation hybrids and only a few back-crossed individuals. Using the 100 % difference criterion, few species-specific markers (i.e. diagnostic loci) were found. Some markers were found with a high frequency in one of the two parental species and low frequency in the other species. Specifically, in *O. incubacea* one marker with a frequency of 100 % and two markers with a frequency of 95 % were found, whereas in *O. iricolor* only one marker with a frequency of 100 % was found.

The mean maximum likelihood (ML) hybrid index scores for individuals of *O. incubacea* and *O. iricolor* was 0·026 (s.e. $\pm$ 0·001) and 0·978 (s.e. $\pm$ 0·001), respectively, and the mean ML hybrid index for hybrid individuals was 0·454 (s.e. $\pm$ 0·007), intermediate between the parental species (Fig. 2).

Patterns revealed by the first two principle co-ordinate axes of the PCA analysis were found to be representative of higher order axes and explain 43 and 27 % of the variation (data not shown). Putative hybrid individuals grouped together in an intermediate position between the parental taxa. AMOVA revealed that 62 % of the variation was found within parental taxa, whereas 38 % of the variation was found between them.

Fruit fitness and hand-pollination experiments

Comparisons of fruit set between *O.incubacea*, *O. iricolor* and hybrids in hybrid zones show non-significant fitness differentiation between parental taxa compared with hybrids (Mann–Whitney *U*-test = 733, $P = 0.835$; Fig. 3). Interspecific crosses conducted on bagged cultivated plants revealed that parental species and hybrid plants differ in the number of viable seeds. In particular, manual crosses revealed that the two parental species produced fruits with a consistent
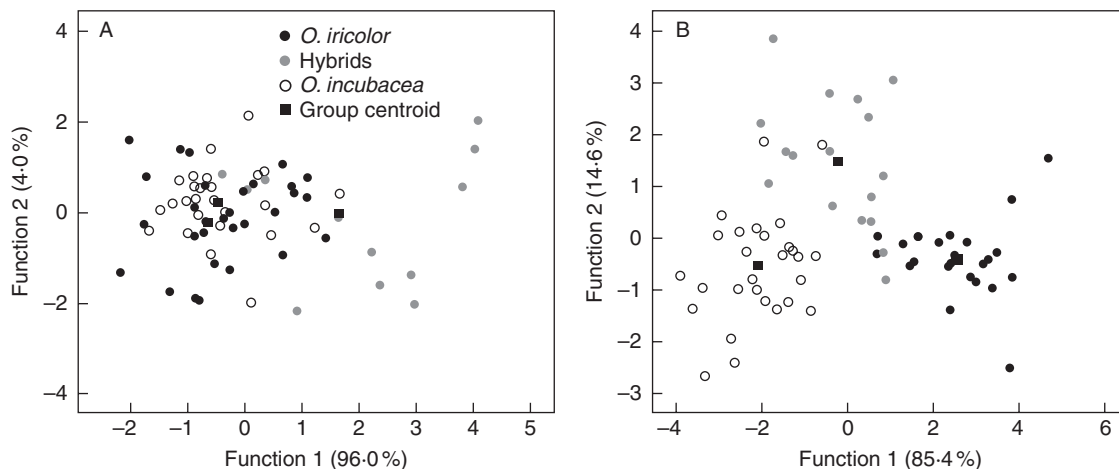

FIG. 1. (A) Floral scent differentiation among taxa in the *Ophrys* hybrid zone. Canonical discriminant function (CDF) plot of all biologically active odour compounds (relative proportions, in %) found in epicuticular extracts of the flowers. Functions 1 and 2 account for 100 % (96·0 and 4·0 %, respectively) of total variability in floral odour among these orchid taxa. (B) Floral scent differentiation among taxa in the *Ophrys* hybrid zone. CDF plot of all non-active odour compounds (relative proportions, in %) found in epicuticular extracts of the flowers. Functions 1 and 2 account for 100 % (85·4 and 14·6 %, respectively) of the total variability in floral odour among these orchid taxa.
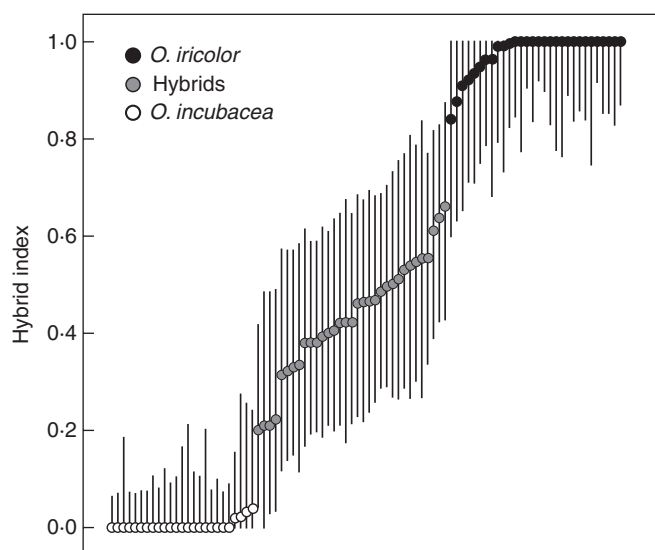
FIG. 2. Molecular hybrid indices ($\pm$ s.d.) for all samples investigated in the hybrid zone. Hybrid indices varying from $0 = Ophrys\ incubacea$ to $1 = O.\ iricolor$. Bars indicate mean and s.d.
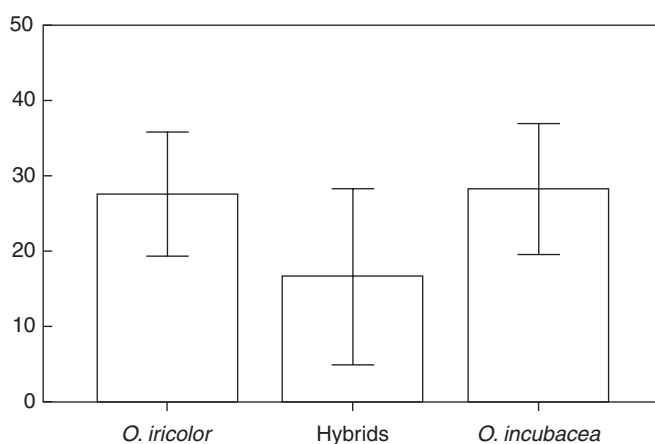

FIG. 3. Percentage of fruit set in *Ophrys iricolor*, hybrids and *O. incubacea* from the hybrid zone. Bars indicate mean and s.e.

TABLE 1. *Percentage of viable seeds (± s.e.) produced from hand pollination of* Ophrys incubacea, O. iricolor *and hybrid individuals in both directions*

Species	*Ophrys incubacea*	Hybrid	*Ophrys iricolor*
Ophrys incubacea	71·5 ± 16·6 % (3)	23·0 ± 8·5 % (6)	72·4 ± 6·2 % (6)
Hybrid		0 % (3)	20·1 ± 8·9 % (4)
Ophrys iricolor			94·2 ± 2·8 % (2)

The numbers of crosses are given in parentheses.

proportion of viable seeds, and F_1 hybrid plants were largely sterile. None of the crosses between hybrid plants produced viable seeds. Manual back-crosses produced fruits with viable seeds mostly when hybrid plants received pollen from parental species, and only a few viable seeds were found when hybrids acted as pollen donors (Table 1).

Micromorphology of Ophrys flowers

The scanning electron microscopy (SEM) analyses revealed marked differences in the labellum morphology of *O. incubacea*, *O. iricolor* and their hybrids (Fig. 4A). The basal region of the labellum of *O. incubacea* was characterized by papillate to acuminate unicellular trichomes with flattened to dome-shaped bases. Lateral lobes of the labellum were raised and showed a transition from short unicellular to long (0.64 ± 0.08 mm) segmented, multicellular and uniseriate trichomes; they were delimited by dome-shaped papillae. The apical region of the labellum showed a rounded appearance with a folded notch that formed a groove delimited by dome-shaped papillae. The groove was flanked by short (0.13 ± 0.05 mm) segmented, multicellular and uniseriate trichomes.

The basal region up to the middle of the labellum of *O. iricolor* was characterized by a central groove and mid-sized (0.20 ± 0.05 mm) filiform, unicellular trichomes obliquely pointing towards the basal groove and stigmatic cavity. Lateral lobes were flattened and showed a transition from short to long (0.56 ± 0.06 mm) unicellular trichomes, delimited by dome-shaped papillae. The apical region was flattened and delimited by dome-shaped papillae. Trichomes appeared unicellular and long (length 0.64 ± 0.12 mm).

The basal region of the labellum of hybrids was characterized by acuminate unicellular trichomes similar to those observed in *O. incubacea*. The lateral lobes were flattened, delimited by dome-shaped papillae and had long, unicellular trichomes reminiscent of *O. iricolor*. The trichomes were rarely segmented, in contrast to what was observed in *O. incubacea*. Frequently, the apical region of the labellum had a lightly rounded appearance and formed a groove. Trichomes appeared unicellular or segmented and long (0.64 ± 0.12 mm; Fig. 4B).

DISCUSSION

By employing a combination of morphological, chemical and molecular approaches, the occurrence of hybridization between *O. incubacea* and *O. iricolor* in Sardinia is unequivocally confirmed. The comparative analyses of parental species and their hybrids in the study area proved particularly useful in the investigation of species boundaries and factors that maintain barriers to gene flow between these two sexually deceptive orchids that share a common pollinator. The results illustrate that hybrids show novel combinations of phenotypic characters in morphology and floral scent (Figs 1 and 2).

Species in sect. *Pseudophrys* are known to differ from members of sect. *Ophrys* in several morphological features of the stigmatic cavity, structure of the labellum and speculum configuration (Devillers and Devillers-Terschuren, 1994). The present micromorphological analyses of flowers show that hybrids combine traits from each parental species: the basal region of their labellum is characterized by acuminate unicellular trichomes as in *O. incubacea*, whereas the lateral lobes of the labella are flattened, and delimited by dome-shaped papillae with long unicellular trichomes similar to those observed in *O. iricolor*. This region of the labellum has been shown to be particularly important in determining the different positions

adopted by pollinators on the labellum during pseudocopulation by guiding the abdomen tip along the basal groove towards the stigmatic cavity. In particular, the direction of lip hairs has been considered crucial for determining whether a pollinator accepts pollinia on the head or abdomen (Kullenberg, 1961; Ågren *et al.*, 1984; Ascensao *et al.*, 2005). However, the finding of an intermediate morphology and orientation of lip hairs in the hybrid plants coupled with the observation of their pollination success (see below) do not strongly support this claim and the role of labellum micromorphology as a reliable mechanical barrier for species isolation.

Patterns of alkanes and alkenes have been found to be divergent between *Ophrys* species attracting different pollinators and almost identical in those species with the same pollinator, thus indicating their importance for pollinator attraction (Schiestl and Ayasse, 2000; Stökl *et al.*, 2005). Convergent evolution of odour signals was found in the case of *O. sphegodes* and *O. fusca* (Schiestl *et al.*, 2000), and more recently it has been reported that flowers of *O. fusca*, *O. sitiaca* and *O. herae*, all pollinated by patrolling males of *A. nigroaenea*, emitted the same biologically active alkanes and alkenes in almost identical proportions. In a cluster analysis performed with these active hydrocarbons or alkenes, *O. fusca*, *O. sitiaca* and *O. herae* always formed a common cluster independent of their phylogenetic relationships (Stökl *et al.*, 2005).

In the present study, it was found that the two parental *Ophrys* species, pollinated by the same bee species, attract their common pollinators by producing similar odour bouquets (Fig. 1A). Biologically active alkanes and alkenes in both species occurred in similar proportions. Hybrids too were shown to produce floral odour bouquets similar to that of their parents (Fig. 1A), although several hybrid samples had a slightly different floral odour signature from that of their parents (Fig. 1A). The multivariate analyses of floral odour differentiation among taxa in active vs. non-active compounds also produced contrasting results: overall, a remarkably lower differentiation among taxa in active compared with non-active odour compounds was found. Such patterns reflect processes of pollinator-imposed convergent evolution in proportions of the BACs in the floral scent of parental species. Since the two parents share the same pollinator species, selection in each species has presumably driven them to evolve similar floral scents that match the sex pheromones of *A. morio* females and odour preferences of *A. morio* males. Likewise, higher differentiation in non-active compounds of the floral odour among taxa in the study species (Fig. 1B) is consistent with theoretical expectation, as compounds that are not involved in pollinator attraction are presumably less subject to selection and hence are more likely to produce contrasting patterns of differentiation (Huber *et al.*, 2005; Mant *et al.*, 2005*b*), especially if the species under study are distantly related. Variation in BACs in hybrids was slightly higher than within parental species (Fig. 1A), which might reflect the consequences of genetic admixture.

Application of molecular markers in this study has revealed a hybrid zone where intermediate hybrid genotypes predominate (Fig. 2). In fact, with few exceptions, most hybrid plants possess a hybrid index value intermediate between those of the parental species, indicating their potential
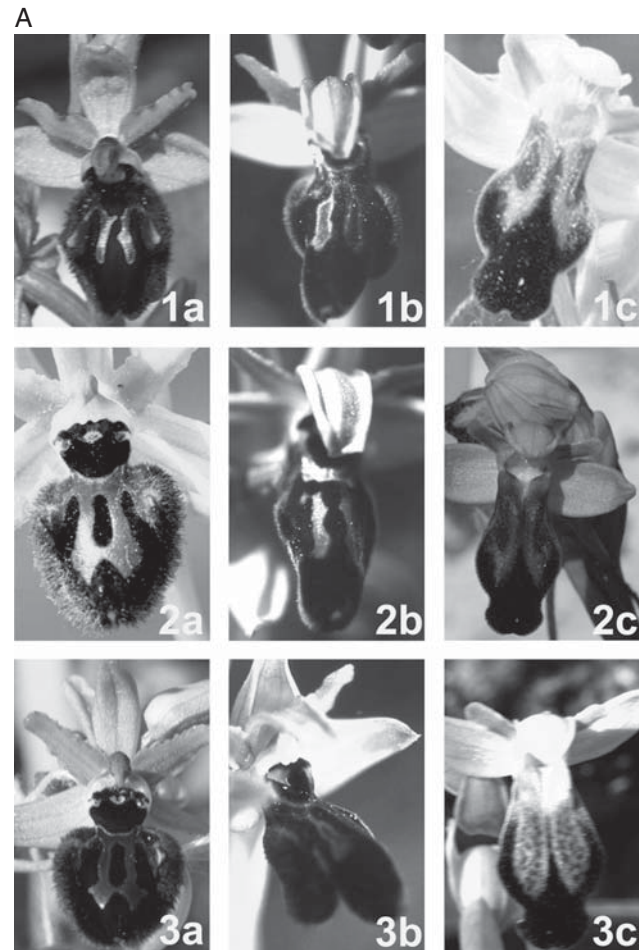

FIG. 4. (A) Flowers and (B) labellum micromorphology of three individuals of *Ophrys incubacea*, *O. iricolor* and their hybrids. Column a, *O. incubacea*; column b, hybrids; and column c, *O. iricolor*. Scales bars in (B) indicate 1 mm in general view and 100 μm in detail.

attribution to the F_1 hybrid genotypic class. This suggests that pre-mating reproductive isolation is incomplete and/or that intrinsic (genetically determined) or extrinsic (environmentally dependent) selection against formation of F_1 hybrids is absent. The apparent ready formation of natural hybrids between species belonging to each section of *Ophrys* (Schlüter, 2006) suggests that differences in pollinia placement between parental species represent an imperfect pre-zygotic isolation barrier. However, at the same time, the rarity of hybrids in other sympatric zones and the low representation of back-crossed individuals in the hybrid zone (Fig. 2) indicate that other (post-mating) selective factors clearly limit interspecific gene flow and maintain species boundaries between the two parental species investigated here.

Estimation of fruit production in sympatric zones can serve as a proxy for plant pollination success (Moccia *et al.*, 2007). The present fitness estimates obtained under natural conditions suggest a reproductive advantage of the parental taxa compared with hybrids, although hybrids are apparently capable of triggering pollinator visitation (Fig. 3). This might at first glance seem surprising, given that hybrids display an original combination of morphological characters compared with their

General view   Detail

B   Basal region
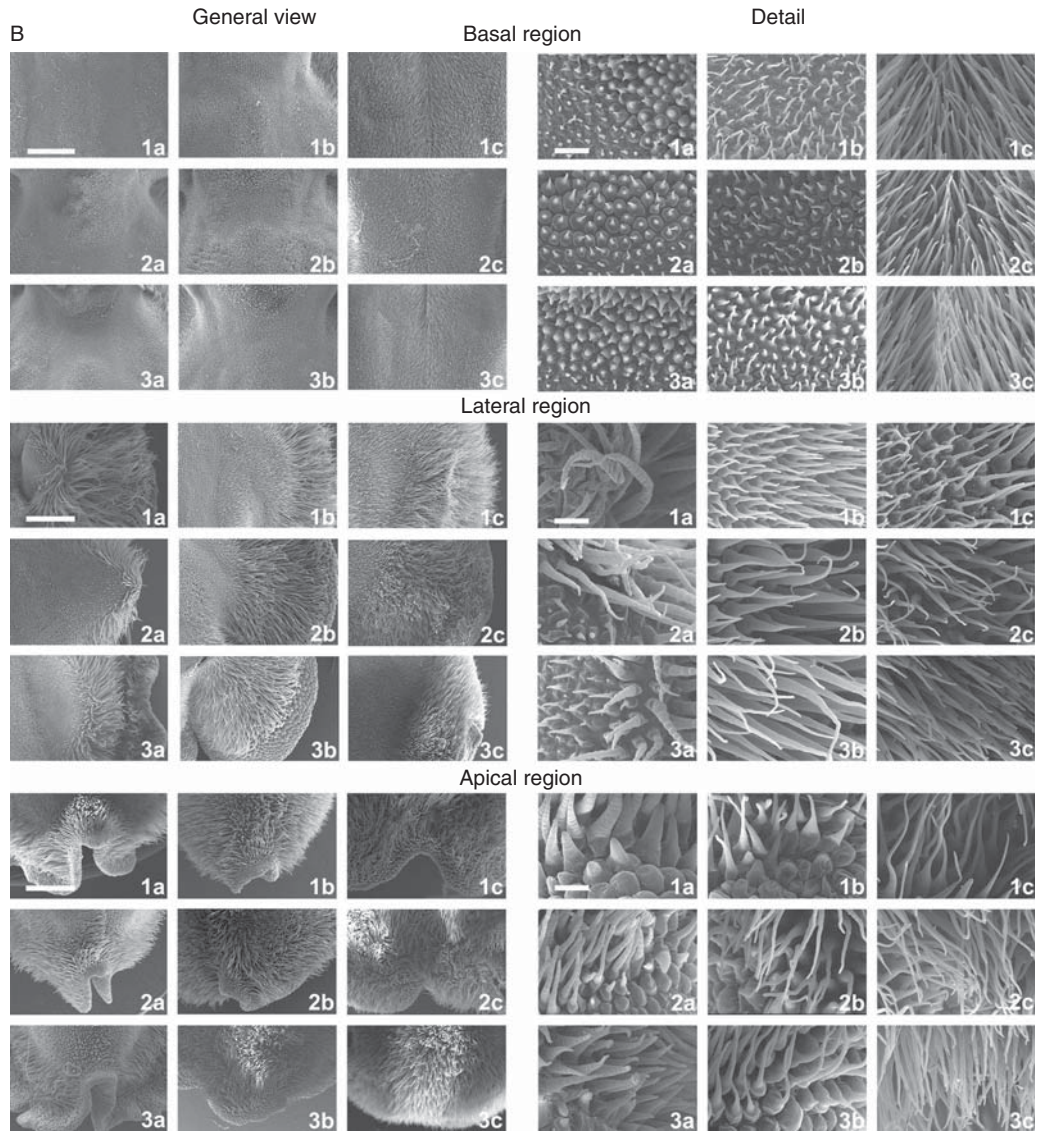
Lateral region

Apical region

FIG. 4. Continued

parental species (Fig. 4A, B) and since floral morphology is expected to play an important role in orienting pollinators. Successful pollination events experienced by hybrids therefore suggest that the differences in floral morphology and scent of hybrids might not exclude pollinator visitations altogether and that pre-mating isolation among species in the hybrid zone investigated remains relatively weak. Field observations of flowers belonging to species in different sections of *Ophrys* (i.e. sect. *Pseudophrys* for the abdomen-pollinated taxa vs. sect. *Ophrys* for the head-pollinated taxa) indicate that pollinators are capable of withdrawing pollinia with both the head and the abdomen during individual pseudocopulation events in members of both sections (N. J. Vereecken *et al.*, unpubl. res.).

A crucial point for hybrid fitness is fertility (Harrison, 1993). In the present study, it has been possible to provide evidence for low fertility through analysis of seed contents in experimental crosses. Hybrids crossed with hybrid pollen failed, whereas parental plants crossed with hybrid pollen produced a few seeds. Only when parental pollen was transferred to hybrids were fruits with a significant proportion of viable seeds produced (Table 1). Thus, the hybrids investigated here produced a significantly lower proportion of viable seeds compared with parental taxa, which led it to be hypothesized that their weak fertility might subsequently reduce formation of second-generation (F_2) or back-crossed individuals. The occurrence of post-zygotic barriers has already been reported in sexually deceptive orchids but generally with a more limited effect than in food-deceptive orchids that share pollinators (Scopece *et al.*, 2007). This reduced F_1 fertility may also explain the genetic architecture of the hybrid zone, with predominantly F_1 and only a few back-crossed individuals.

Recent karyological analyses (D'Emerico *et al.*, 2005) on the same orchid populations showed that parental species have the same chromosome number ($2n = 36$), which rules out a diploid/tetraploid hybridization scenario. However, in

contrast to *O. iricolor*, *O. incubacea* showed a more asymmetrical karyotype with several sub-metacentric chromosomes; the intrachromosomal asymmetry index was 0·26 ($\pm$0·01) for *O. iricolor* and 0·32 ($\pm$0·03) for *O. incubacea*, respectively (D'Emerico *et al.*, 2005), indicating that several chromosomal rearrangements have occurred (Cozzolino *et al.*, 2004). In crosses between chromosomally divergent species, reduced F_1 fertility or sterility is often attributed to the effects of chromosomal rearrangements on meiotic pairing (Stebbins, 1971; Rieseberg, 2001). As a consequence, karyotype differences between parental species may be the reason why most hybrids have been found to be F_1 (Fig. 2) and have highly reduced fertility (Table 1), which is consistent with the genetic pattern observed in the hybrid zone investigated here and in similar studies on food-deceptive orchids that have low pollinator specificity (Moccia *et al.*, 2007; Scopece *et al.*, 2007, 2008). Consequently, even in a group such as sexually deceptive *Ophrys* with a highly specific pollination mechanism, karyotype differences can cause the observed post-zygotic reproductive isolation, and these differences can consequently contribute to maintenance of species boundaries in secondary contact zones of *Ophrys* species that share a common pollinator.

The situation observed here allows identification of parallels with other sexually deceptive orchid systems. The Australian *Chiloglottis trapeziformis* and *C. valida* are pollinated by the thynnine wasps *Neozeleboria cryptoides* and *N. monticola*, respectively, by using the same attractive sex pheromone (i.e. chiloglottone). Their hybrid, *Chiloglottis × pescottiana*, was revealed to be mainly F_1 and displayed reduced pollen viability and seed set, thus suggesting occurrence of post-zygotic barriers (Peakall *et al.*, 1997).

In this context, the present study provides a new perspective on the role of pre-mating reproductive isolation in Mediterranean sexually deceptive orchids, which have so far been assumed to rely primarily on pre-mating isolation, whereas post-mating isolation was thought to make little contribution to maintenance of species boundaries (Ehrendorfer, 1980). Indeed, similar ongoing studies in hybrid zones between *Ophrys* species that attract different, specific pollinators revealed occurrence of high levels of interspecific gene flow and introgression, as expected in a system with little post-mating isolation (Stökl *et al.*, 2008).

Even if F_1 hybrids suffer large reductions in fitness due to intrinsic (genetically based) selection, the evolutionary consequences of hybridization may still be significant in these sexually deceptive orchids. Hybrids, by emitting original blends of pollinator-attracting odour compounds, could gain access to a novel pollinator (N. J. Vereecken *et al.*, unpubl. res.). However, in this study, both parental species produced similar floral odour bouquets, and hybrids thus do not differ significantly in their scent emission. This might be the direct consequence of parental species using similar enzymatic pathways to produce analogous floral odour bouquets, which makes interactions among the parental alleles involved in scent production possible even within the hybrid genomic context. In this special circumstance, hybrid phenotypes do not appear to have the ability to attract a novel pollinator as suggested in the textbook cases on homoploid speciation (Arnold, 1997).

Recent molecular phylogenetic studies have shown that the species of sect. *Pseudophrys* form a clade (Soliva *et al.*, 2001; Bateman *et al.*, 2003; Devey *et al.*, 2007), which suggests that evolution of an 'inverted' labellum was a unique event in development of the *Ophrys* flower. The unique origin of the *Pseudophrys* lineage suggests that the occurrence of 'inverted' mutants might have provided the basis for reproductive isolation and fostered speciation in this group of orchids, especially if other barriers or geographic isolation occurred in the early phase of species differentiation when pollinators are initially shared. The finding of a marked difference in chromosomal patterns in these *Ophrys* species suggests that karyotype differences evolved in allopatry may have also played an important role in species formation and maintenance of species boundaries. The genus *Ophrys* evolved in a highly fragmented area of the centre of the Mediterranean basin, between Southern Italy and Greece, under conditions that are likely to have favoured the fixation of mutations through genetic drift and inbreeding (Levin, 2002). Hence, the combined effects of chromosomal rearrangements and labellum inversion might have constituted an unusual conjunction of events that might then have fuelled future speciation of species in sect. *Pseudophrys*.

After the initial formation of the *Pseudophrys* lineage, thanks to reproductive isolation from widespread sect. *Ophrys*, it may have rapidly exploited a range of pollinators and radiated. Changes in floral odour and associated pollinator switches are considered the main cause of speciation in *Ophrys*, and several putative case studies have been reported among different *Pseudophrys* species complexes (Schiestl and Ayasse, 2002). However, the secondary co-existence of cephalic/abdominal *Ophrys* species sharing the same pollinator may also have triggered switches to novel pollinators, a strategy that might have helped avoid gamete wastage in hybridization, a consequence of imperfect mechanical isolation. Under these circumstances, pollinator switches would represent the extreme consequence of character displacement (*sensu* Butlin, 1997) rather than the initial outcome of the speciation process *per se*.

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