

Morphological and molecular investigation of the parentage of *Ophrys* × *circlarium* (*O. lutea* × *O. tarentina*), a new hybrid orchid from Italy

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This study reports a new hybrid combination *Ophrys* × *circlarium* Pellegrino, *hybr. nov.*, which derives from two highly divergent species, *O. lutea* (*O. fusca-lutea* complex) and *O. tarentina* (*O. sphegodes* complex). These two species grow sympatrically in the north of Calabria region (southern Italy), in a stand where two potential hybrid individuals were found during a floristic investigation. Two of the 16 morphometric characters analyzed were intermediate relative to those of the potential parental species. PCR-RFLP analysis of nuclear ribosomal DNA Internal Transcribed Spacer sequences (rDNA ITS) confirmed that the two specimens are hybrids of the two co-occurring *Ophrys* species.

Key words: ITS, new hybrids, *Ophrys lutea*, *Ophrys tarentina*, rDNA

Introduction

Natural hybridization among vascular plants is a relatively common phenomenon and has played a significant role in their evolution (Grant 1981). Although it is not clear whether spontaneous hybrid formation is a typical feature of some plant groups, it is likely that interbreeding might be frequent among still divergent taxa and, thus, within the more recent and advanced families of the angiosperms. In this respect, a suitable example is represented by the Mediterranean food deceptive orchids, like *Orchis*, *Anacamptis* and *Dactylorhiza*, in which frequent hybridization has been documented (Delforge 2001) and attributed to their unspecific pollination system

(Van der Cingel 1995).

Noticeably, even within the sexually deceptive genus *Ophrys*, known for its peculiar pollinator specificity, interbreeding is frequent (Nelson 1962, Danesch & Danesch 1972, Baumann & Künkele 1982, Delforge 2001) and likely contributes to the difficulties in the taxonomy. Indeed, the number of *Ophrys* species is on the increase (21, 68, 148, 215 or 250 species according to Nelson 1962, Baumann & Künkele 1988, Delforge 1994, 2001, 2005, respectively) and, in the course of time, several classical binomial combinations, such as *O. bertolonii*, *O. fuciflora*, *O. sphegodes* and *O. fusca*, have become aggregates of numerous taxa, weakly differing in their morphological traits, flowering period

and pollinators. In addition, hybridization events between *Ophrys* aggregates may be facilitated by their shared ploidy level ($2n = 36$) (Greilhuber & Ehrendorfer 1975, Bianco *et al.* 1989).

In the course of a floristic investigation in the northern Calabria region, the authors visited a site where *Ophrys tarentina* was found sympatric with *O. lutea*. At this site, two orchid specimens were noticed for their strikingly unusual flowers, which appeared to combine traits of the two co-occurring species and thus were suspected to be their hybrid progeny. This finding was unexpected because the two presumed parental orchid species possess different pollination strategies and belong to different sections of *Ophrys* (Godfery 1928, Delforge 1994). *Ophrys tarentina* belongs to the section *Euophrys* and its pollinators are bees of the genus *Osmia*, which may carry pollinaria on their head. *Ophrys lutea* on the other hand belongs to the section *Pseudophrys* and is pollinated by several species of the bee genus *Andrena*, which remove pollinaria with their abdomen (Paulus & Gack 1990a, 1990b, 1990c).

We considered this finding worthy of reporting and sampled the presumed hybrid and both parent species to facilitate subsequent morphometric and molecular investigations. Molecular approaches have successfully been applied to study taxonomic position and parental lineage of hybrid specimens as well as to characterize gene flow in hybrid zones (Bateman & Hollingsworth 2004, Pellegrino *et al.* 2005). For *Ophrys*, a molecular study of presumed hybrids was undertaken only once (Gulyás *et al.* 2005) to the best of our knowledge. Therefore, the main goals of this study were (1) to furnish a morphological description of the relevant features of the hybrid plants in comparison with the co-occurring *Ophrys* species, (2) to ascertain with molecular analysis the hybrid origin of the two specimens, and (3) to correctly identify their parental species.

Material and methods

The site hosting *O. tarentina*, *O. lutea* and their presumed hybrid was visited on 20 April 2005. It is located along the right-hand side of the road

(SS92), a few kilometres north of Cerchiara di Calabria (northern Calabria region, Italy). The two hybrid flower spikes were likely emerging from a single rootstock suggesting that the smaller one was a recent clonal derivative of the other (Fig. 1). No further similar specimens were detected in the vicinity and thus we did not collect any of the two plants for herbarium.

To minimise disturbance of the orchids, vegetative and floral measurements were made in the field only on the more phenologically advanced individual of the two hybrids and on ten average-sized individuals of each putative parent. For each specimen, eight qualitative and eight quantitative diagnostic floral traits (Table 1) were evaluated on the second flower from the bottom of the inflorescence. Quantitative measures were made to the nearest 1 mm using a ruler.

For molecular analysis, one fresh cauline leaf of both presumed hybrids and three specimens of each sympatric *Ophrys* species were sampled and stored in silica gel. For total DNA extraction approx. 4 cm² of each leaf were separately pestled in a 2-ml eppendorf using 500 µl of isolation buffer (2% hexadecyltrimethyl ammonium bromide). Successive procedures were in accordance with Doyle and Doyle (1987) protocol. To determine whether the intermediate plants were actually of hybrid origin, rDNA Internal Transcribed Spacer sequences were obtained from the putative parental taxa. The internal ribosomal spacers (ITS 1 and ITS 2) were amplified with polymerase chain reaction (PCR). The ITS1 was amplified using a pair of primers, which anneal in the 3' region of the 18S (5'-GAGAAGTCGTAACAAGGTTTCCG-3') and in the 5' region of the 5.8S (5'-ATCCTGCAATTCACACCAAGTATCG-3'). The ITS2 region was amplified using a pair of primers annealing in the 3' region of the 5.8S (5'-TTGCAGAATCCGTGAACCATCG-3') and in the 5' region of the 25S (5'-CCAAACAACCCGACTCGTAGACAGC-3'). All PCR reactions of 100 µl final volume contained 2 µl DNA template, 100 µM of each dNTP, 0.3 µM of each primer, 2 units Taq polymerase, 2 µM MgCl₂ and 10 µl reaction buffer.

PCR reactions were conducted in a thermal cycle (Perkin Elmer 2600) for 30 cycles. Initial conditions were as follows: 30 sec denaturation



Fig. 1. *Ophrys* × *circlarium* and its parents. — **A:** The two inflorescences of *O. × circlarium*. — **B:** Flowers of *O. lutea*. — **C:** Flower of *O. × circlarium*. — **D:** Flower of *O. tarentina*. All taken at the Cerchiara di Calabria, Italy.

at 94 °C, 1 min annealing at 55 °C, 45 sec extension at 72 °C; extension time was increased by 3 sec/cycle; extension was further prolonged for 7 min at the end of the last cycle. Amplified ITSs were electrophoretically separated on a 2% agarose gel. A 100 base pair (bp) ladder (Pharmacia Biotech) was used as a molecular weight marker.

Amplified fragments were sequenced in both directions using a modification of the Sanger dideoxy method as implemented in a double stranded DNA cycle sequencing system with fluorescent dyes. Sequence reactions were then run on a 373A Applied Biosystems Automated

DNA sequencer (Applied Biosystems, Foster City, CA, USA).

The sequences were examined using Gene-Jockey to find a restriction site that would distinguish them using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). This approach allows the examination of a heterozygous individual (e.g., a hybrid) without the necessity of cloning and subsequently sequencing several ITS clones (heterozygous individuals give overlapping traces from direct sequencing that are often difficult to interpret). Restriction enzyme *Mae*III, which cuts at GTNAC, differentiated the putative parental taxa

due to the presence of a G/A substitution about 77 base pairs into the ITS 1 sequence (GTTGC in *O. lutea*, GTTAC in *O. tarentina*); while *AluI*, which cuts at AGCT, showed two nucleotide substitution C/T, about 154 base pairs (AGCT in *O. lutea*, AGTT in *O. tarentina*), and G/T, about 203 base pairs (AGCT in *O. lutea*, ATCT in *O. tarentina*) into the ITS 2 sequences.

Fragments of hybrids and parental species (100 ng) were then digested in a final volume of 20 µl with selected restriction endonucleases (1U/µg DNA), according to the manufacturer's instructions (Fermentas) and electrophoretically separated on a 3% agarose gel (Metaphore agarose FMC, U.S.A), using a 100 base pair (bp) ladder (Pharmacia Biotech) as molecular weight marker, stained with ethidium bromide and photographed using a Kodak digital camera.

Results

Morphological analysis

Morphological observations confirmed the initial, visual impression that the presumed hybrid

plants exhibited six features typical of one or the other co-occurring *Ophrys* species, two characters are more or less intermediate, while two other characters lie outside the values of both parents (Table 1).

As regards a structure of diagnostic value, the labellum of the hybrid plants is 3-lobed and marginally yellow as that of *O. lutea*, while its H-shaped speculum looks like that of *O. tarentina*. The qualitative features of the sepals and lateral petals are essentially those observed in *O. tarentina*. (Table 1), except the slight curvature of the median sepal similar to that of *O. lutea* (Table 1).

Regarding the size of vegetative and floral traits, we found that petal dimensions (length and width) of the hybrid are shifted toward the range variation detected in one or the other of the two parental species, while hybrid labellum is smaller than those of its parental species (Table 1).

Molecular analysis

The ITS-containing fragments obtained from the three taxa were approximately 280 (ITS 1)

Table 1. Morphometric comparisons of *Ophrys lutea*, *O. × xcirclarium* and *O. tarentina*. Hybrid features intermediate in respect to those of the parents are set in boldface, those typical of one or the other parental *Ophrys* species are set in italics.

Morphological traits	<i>O. lutea</i> (n = 10)	<i>O. xcirclarium</i> (n = 1)	<i>O. tarentina</i> (n = 10)
Plant height (cm)	18 ± 1.8	24	27 ± 2.2
Spike	lax	lax	lax
No. of flowers	5 ± 0.5	4	7 ± 0.5
Basal leaf	ovate, acute	ovate, obtuse	ovate-lanceolate, obtuse
Lateral sepal length (mm)	9 ± 0.2	9	10 ± 0.5
Lateral sepal width (mm)	6 ± 0.2	6	6 ± 0.4
Lateral sepal	green-yellowish, sub-patent	green, <i>oblong-ovate</i>	green, <i>oblong-ovate</i> to lanceolate
Median sepal	green-yellowish, <i>incurved</i>	green, weakly <i>incurved</i> , <i>narrower than laterals</i>	green, <i>narrower than laterals</i>
Petal length (mm)	7 ± 0.3	8	10 ± 0.6
Petal width (mm)	2 ± 0.2	3	3.5 ± 0.3
Petal	green-yellowish, linear-oblong	green, <i>lanceolate</i>	green, <i>oblong-triangular to lanceolate</i>
Lip shape	<i>3-lobed</i> , lateral ovate; middle reniform	<i>weakly 3-lobed</i> ,	entire
Lip length (mm)	16 ± 0.6	10	12 ± 0.6
Lip width (mm)	14 ± 0.4	11	15 ± 0.8
Lip marginal zone (mm)	2.5 ± 0.1, <i>glabrous, yellow</i>	3, <i>glabrous, yellow</i>	3 ± 0.1, hairy, brown
Speculum	2-lobed, bluish-grey	<i>H-shaped intricate, bluish-violet</i>	<i>H-shaped</i> without cross-line, <i>bluish-violet</i>

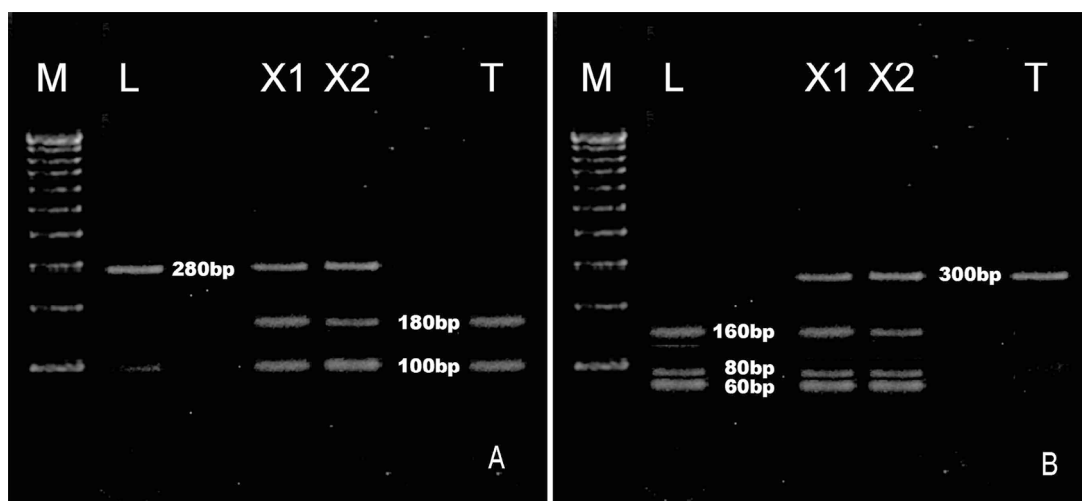


Fig. 2. (A) Gel electrophoresis of ITS 1 *Mae* III digestions and (B) ITS 2 *Alu* I digestions showing the presence of characteristic fragments of the ITS region of *Ophrys lutea* (lane L), *O. × circlarium* (lanes X1–X2) and *O. tarentina* (lane T). Molecular 100 bp ladder (line M). The hybrids show an additive profile of the contrasting RFLP patterns derived from the two parental species.

and 300 (ITS 2) bp in length. As expected, the known ITS divergence allowed to yield different, diagnostic restriction profiles for the two putative parental species. Indeed, ITS 1-containing fragments digested with *Mae*III showed a single restriction site in *O. tarentina* (with two fragments approx. 180 bp and 100 bp long) and no site in *O. lutea* (Fig. 2A). The ITS 2-containing fragments digested with *Alu* I showed two restriction sites in *O. lutea* (with three fragments approx. 160 bp, 80 bp and 60 bp long) and no site in *O. tarentina* (Fig. 2B).

The hybrid plants exhibited a direct additive inheritance of these profiles, in the sense that their digested fragments produced the combination of diagnostic profiles obtained for both *O. lutea* and *O. tarentina* (Fig. 2). Therefore, the molecular analysis yielded strong support for the hybrid origin of our specimens from the two co-occurring *Ophrys* species.

Discussion

In this study morphological and molecular results unequivocally demonstrated that the two discovered plants are the natural hybrids between *O. tarentina* and *O. lutea*. To our knowledge, this is the first report of the interbreeding capacity of

these two species, although several other hybrids among taxa of the *O. lutea* and *O. sphegodes* groups have been recognized on morphological basis. This novel hybrid combination is here named *O. × circlarium*, with reference to the ancient name of Cerchiara di Calabria, the city nearest to the site of finding.

Nowadays, there is a renewed interest in the study of plant hybridization due to the awareness of its evolutionary implications. Indeed, application of molecular methods alongside with experimental crosses, have explained some events of hetero- and homoploid hybrid speciation (Rieseberg 1991, Soltis & Soltis 1999, Buerkle *et al.* 2000). Also, it is now clear that hybrid swarms are more variable than expected and may represent a source of novel adaptations (Rieseberg & Wendel 1993).

The extraordinary species richness of the Orchidaceae has long been thought to have arisen as a consequence of the high pollinator specificity acting as a mechanism of premating reproductive barrier, an hypothesis corroborated by the compatibility observed in experimental crosses also among taxa with low phylogenetic relatedness (van der Pijl & Dodson 1966). However, the unexpected high level of natural hybrids found among the Mediterranean orchids inspired accurate investigations on their pollina-

tion strategies (van der Cingel 1995). As a consequence, a still open debate originated on the role of hybridization in the evolution of the Mediterranean orchids, the type and effectiveness of the reproductive barriers and the conservation value of these orchid hybrids and hybrid zones (Cozzolino *et al.* 2006).

Phylogenetic evidence shows that speciation of Mediterranean orchids is not strictly associated with shifts in flower morphology, coloration or scents, that usually represent evolutionary innovation keys (Cozzolino & Widmer 2005), but may be produced by hybridization events. In addition, hybrid swarms consist prevalently of F1 individuals, while introgressive and later hybrid generation specimens are rare or absent, suggesting the occurrence of post-zygotic reproductive barriers (Cozzolino *et al.* 2006), such as asymmetrical karyotypes (Cozzolino *et al.* 2004).

In any case, hybridization in orchids appears to be not a secondary effect of habitat disturbance as is usual for other plant groups, but is rather a natural consequence of their pollination system and linked to a secondary contact of previously isolated taxa. This implies that orchid hybrids and hybrid zones, at least in the Mediterranean region, might be seen as a stage for evolutionary processes and that relative conservation strategies will be adequately conceived (Cozzolino *et al.* 2006).

Moreover, molecular characterization of hybrids may provide valuable evidence for the genetic delimitation of evolving lineages, as illustrated by the study on the parentage and maternity of *Anacamptis xalbuferensis*, a new hybrid combination between two divergent *Anacamptis* species groups (Bateman & Hollingsworth 2004). According to those authors, this and other records of hybridization among *Anacamptis* groups reflect their similar habitat preferences, overlapping flowering times, and their genetic compatibility, thus corroborating the splitting of these lineages from the genus *Orchis* (Bateman *et al.* 2003).

Recently, a phylogenetic analysis of the genus *Ophrys*, based on nuclear and chloroplast DNA sequences, has confirmed the genetic divergence of the two sections *Pseudophrys* and *Euophrys* (Bateman *et al.* 2003), traditionally recognized on the basis of their labellum differences and

mechanism of pollinaria removal (Godfrey 1928). Unfortunately, this analysis did not fully resolve the species relationships, suggesting a recent radiation of the genus and a potential role of hybridization in its evolution (Soliva *et al.* 2001). Hence, evidence from hybrids may help to better understand the genetic cohesion of *Ophrys* groups. However, this and other orchid genera, such as *Dactylorhiza*, *Epipactis* and *Serapias*, have insofar exhibited a very low difference of the suitable nuclear sequences, presumably due to a very recent divergence of their lineages.

In any case, any hybrid combination is worthy to be reported because it could represent not only a taxonomical novelty or a merely botanical curiosity, but a further evidence to better understanding the evolutionary history of a given plant group.

Diagnosis

Ophrys × *circlarium* Pellegrino, *hybr. nov.* (Fig. 1)

Hybrida statura mediocri (24 cm) *inflorescentia laxa floribus*. *Sepala lateralalia viridia ovata*, 9 mm longa, 6 mm lata. *Sepalum medium supra columnam inclinatum, minus latum quam sepala lateralalia*. *Petala lateralalia viridia lanceolata*, 8 mm longa, 3 mm lata. *Labellum parvum subrotundatum trilobum*, 10 mm longum, 11 mm latum, *speculum forma H contorta*. *Hybrida plurimis formis inter species parentales intermediis*. *Ophrys lutea simillima, labello trilobato margine luteo*. *O. tarentina simillima, sepala lateralalia ovata, petala lanceolata*.

HOLOTYPE: Italy. Along road to Cerchiara di Calabria, 39°51'N, 16°23'E, (Calabria region), 18.IV.2005, Pellegrino *et al.* s.n. (CLU).

Morphological characters more or less intermediate between the parental species *O. lutea* and *O. tarentina*. Closely resembles *O. lutea* by possessing a tri-lobed lip with yellow marginal zone, and closely resembles *O. tarentina* by lateral sepals oblong-ovate and petals lanceolate, and labellum with H-shaped intricate speculum.

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