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A small hybridization flow between *Orchis collina* and *O. longicornu* in Sicily

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Riassunto. *Un ridotto flusso di ibridazione tra* Orchis collina *e* Orchis longicornu *in Sicilia.*
Sono state utilizzate le sequenze spaziatrici del DNA nucleare ribosomale per indagare l'origine ibridogena di sei presunti ibridi tra *Orchis collina* e *Orchis longicornu* trovati in Sicilia. Gli individui esaminati risultano possedere il DNA ribosomale di entrambe le specie parentali, confermando così la loro natura ibridogena. I presenti risultati confermano la possibile, anche se rara, presenza di fenomeni di ibridazione tra *O. collina* e *O. longicornu* in una zona dove le due specie crescono simpatricamente e a dispetto del fatto che apparentemente adottino differenti strategie di impollinazione.

Key words: Hybrids, ITS, Orchids, rDNA,

INTRODUCTION

Sympatry in phylogenetically related orchid species may occur because efficacious reproductive barriers have developed which prevent large hybridisation phenomena. However in the orchid family hybrids have been often found in nature (DANESH & DANESH, 1972) and their presence and occurrence may represent an indication of the level of genetic barriers in the taxa. In genus *Orchis*, the limit to the occurrence of extensive gene flow among species seems to be represented mainly by ethological or mechanical barriers rather than having a genetic basis (ACETO *et al.*, 1999a; PELLEGRINO *et al.*, 2000).

In this study we focus on a pair of species closely related from a morphological and a phylogenetic point of view (PRIDGEON *et al.*, 1997; ACETO *et al.*, 1999b), *O. collina* Solander and *O. longicornu*

Poiret, which grow sympatrically only in some areas of Sicily, where the two distribution areas overlap (GALESI, 1995). In one of these areas, intermediate phenotypes between these species have been found and classified as *O.* x*santamariotae* Galesi and Grasso (GALESI, 1995; GALESI & GRASSO, 1992).

Information on the pollination biology of the parental species is scanty. Typically, unrewarding *Orchis* species attract pollinators imitating floral shape and colour of nectar-producing plants (VAN DER CINGEL, 1995). As the closely related and widespread *O. morio, O. longicornu*, employing this strategy, is pollinated mainly by naive bumble bee queens and solitary bees which visit the showy flowers while searching for nectar after hibernation (NILSSON, 1984).

A similar pollination strategy, i.e. food deception, has also been reported for *O. collina*. However, in contrast to most other food-deceptive taxa as *O. longicornu, O. collina* strictly mimics only the closely related, nectariferous *O. coriophora*. In this way, *O. collina* is pollinated by the same insect species that also visit *O. coriophora* (DAFNI & IVRY, 1979)

In this regard it seems unusual that, despite apparent differences in pollination preferences of the two species, there is evidence of hybrids between *O. collina* and *O. longicornu*. For this reason a molecular approach appears to be a necessary tool to assure a correct detection of hybrid status.

In the present paper, the authors investigate some putative hybrids between *O. collina* and *O. longicornu*, in order to characterise the found specimen from a molecular point of view, as well as to ensure the correct identification of their parental species.

MATERIALS AND METHODS

In the course of a floristic investigation in the Natural Reserve "Sughereta di Niscemi" (Caltanissetta, Sicily, Southern Italy), at the end of February 2000, in a meadow with *Quercus suber* L., the authors found six flowering orchid specimens showing intermedia-

te morphology between *O. collina* and *O. longicornu*, and therefore attributed to *O.* x*santamariotae*

Fig. 1 - A specimen of *O.* x*santamariotae*

The three taxa are shifted in flowering time; the six investigated specimens blooming in the intermediate period between *O. collina* (from the end of January to the middle of February) and *O. longicornu* (from the beginning to the end of March) flowering times. None other orchid have been found flowering in the same period in this area; at the same time, however, many different species growing sympatrically with the investigated specimen were in flower. Among, these *Asphodelus microcarpus* Viv., *Pistacia lentiscus* L. and *Cistus incanus* L. were the most abundant. Voucher specimens

of all investigated orchid taxa have been deposited at Herbarium of University of Naples Federico II (NAP).

Total DNA was extracted according to DOYLE & DOYLE (1991) from 0.05-0.1 g of fresh leaves. The ITS2 region was amplified by polymerase chain reaction (PCR) using primers annealing with the 3' region of the 5.8S (5'-TTGCAGAATCCCGTGAACCATCG-3') and the 5' region of the 25S (5'-CCAAACAACCCGACTCGTA-GACAGC-3') rDNA genes, by using the conditions described in ACETO *et al.* (1999b). All amplified fragments were purified using Microcon 100 microconcentrators (Amicon, MWCO 100,000) with minor modifications of the manufacturer's protocol. Two washes with 450 ml TE buffer (10mM Tris-HCl, pH 7.5, 1mM EDTA) were carried out by centrifugation at 7000g for 6 min to eliminate any salt residue. Purified fragments were then digested with the restriction endonucleases *Hinf*I, electrophoretically separated on a 2% agarose gel stained with ethidium bromide and photographed on a UV trans-illuminator. A 100 base pair (bp) ladder (Pharmacia Biotech) was used as molecular weight marker.

RESULTS

The found specimens possessed some morphological traits which were intermediate between the putative parental species. In particular, the purple-pinkish labellum, almost trilobate, with reflexed lateral lobes and whitish median lobe with or without purple dots, the cylindrical spur, horizontal and almost as long as the ovary, and the size of floral bracts represent intermediate features between the floral shapes of parental taxa.

The observation of the ITS2-containing fragments has shown no length differences between the parental species in the amplified regions.

The ITS2-containing fragments obtained from *O. collina* and *O. longicornu* were approx. 600 bp in length. With the intent of discriminating among sequences, a restriction analysis was carried out. For ITS2, the choice of the restriction enzyme was aimed at choos-

ing sites which were different between parents. Parental ITS2-containing fragments digested with *Hinf*I showed a single restriction site in *O. collina* (with fragments approx. 410 bp and 150 bp long) and two restriction sites in *O. longicornu* (with fragments approx. 250 bp, 160 bp and 150 bp long). In fact, in *O. longicornu* an additional *Hinf*I restriction site appears in the 450 bp long fragment, resulting in a 250 bp and a 160 bp long fragments. The ITS2-containing fragments of all the hybrids, when digested with *HinfI*, showed the presence of four distinct bands (with fragments approx. 410 bp, 250 bp, 160bp and 150 bp long), corresponding in size to the fragments resulting from the digestions of *O. collina* and *O. longicornu.*

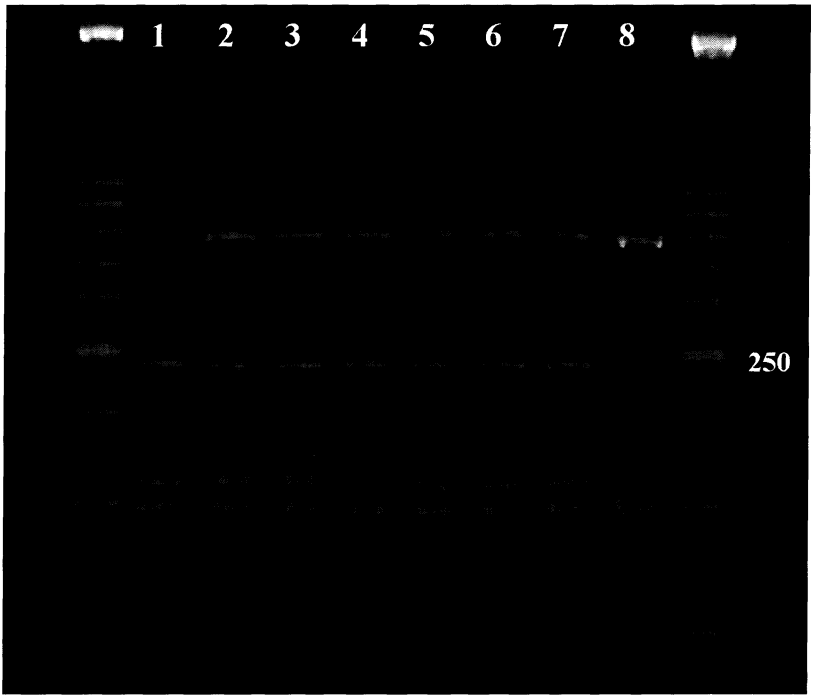

Fig. 2 - Gel electrophoresis of the ITS2 *Hinf*I digestion of *O. collina* (line 2), six specimens of *O.* x*santamariotae* (from line 3 to line 8), *O. longicornu* (line 9), and molecular 100 bp ladder (line 1 and line 10).

Discussion

Morphological observation of the found specimens indicates that some floral characters appear intermediate between the hypothesised parental species; the identification of hybrid individuals, however, may often be difficult when they resemble more one parent than the other or when new morphological combinations of characters arise from recombination of distinct genotypes. In this respect, the molecular approach may represent an useful tool for the assessment of hybrid status and for the correct identification of parental lineages.

ITS restriction patterns of the hybrids, in which all the exclusive parental bands are always present, clearly indicate that *O. collina* and *O. longicornu* provide parental lineages as previously postulated on morphological bases.

In the present circumstances, with the hypothesised parental species normally not hybridising, a combined approach allows to avoid any doubt on the real hybrid status of *O.* x*santamariotae* and on the correct identification of parental species.

The identification of parental species also excludes the possibility that other species may be involved in the formation of this hybrid and clearly demonstrates that a reduced hybrid production occurs between *O. collina* and *O. longicornu*, even if they have different pollination aptitudes and flowering times which do not overlap. The phenotypic variability of the hybrids and the presence of mature pollinaria and seed capsules, suggesting hybrid fertility and possible introgression, indicate that probably no (or at least weak) post-zigotic barriers exist between *O. collina* and *O. longicornu* and that presumably only strong ethological isolation grants preservation of species integrity, only allowing minimal gene flow between the two taxa.

Due to dearth of information on local pollination aptitudes of the parental species, only mere speculations may be made in order to explain the occurrence, even if reduced, of gene flow among the two taxa. Probably, the absence of *O. coriophora* (i.e. the presumed model employed by *O. collina* to obtain pollination) in the place

where *O. collina* and *O. longicornu* grow simpatrically allows *O. collina* to adopt a different pollinator set in this locality (i.e. different pollinators from those typically employed by *O. collina* when it grows together with *O. coriophora*). In this circumstance, a small overlap in the pollinator set with *O. longicornu*, which usually starts flowering two weeks later, may have occurred in the area where the two species grow simpatrically and the hybrid specimens have been found. Probably, some insects still carrying pollinaria of late flowering *O. collina* visited the first flowering individuals of *O. longicornu*, so allowing hybridisation.

However the low frequency of hybrid finding is an indication that also in the absence of its preferential model, *O. collina* generally adopts pollinators and phenology largely different from those of *O. longicornu*, and the finding of few hybrids, even if confirming the absence of genetic barriers between these species, may be considered only as an unusual event.

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Abstract.

The Internal Transcribed Spacer sequences of the nuclear ribosomal DNA (rDNA ITS) were used to verify hybrid origin of six putative hybrids between *Orchis collina* and *O. longicornu* found in Sicily. The investigated specimens possessed ribosomal DNA from both the hypothesized parental species thus confirming their hybrid nature. Present results confirm the possible, even if rare, occurrence of hybridisation between *O. collina* and *O. longicornu* in an area where they grow simpatrically in spite of the fact that they apparently have different pollination strategies.

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