Molecular characterization of a hybrid zone between *Orchis mascula* and *O. pauciflora* in Southern Italy

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Abstract

A small population (16 individuals) of *Orchis* × *colemanii* Cortesi, a natural hybrid between *O. mascula* (L.) L. and *O. pauciflora* Ten. was sampled from a locality near Sassano (National Park of Cilento, province of Salerno, Southern Italy) in order to assess its genetic structure. Filter hybridizations of *O.* × *colemanii*, *O. mascula* and *O. pauciflora* DNAs against a PCR-amplified ribosomal fragment indicated that all the hybrid individuals in study have the ribosomal repeats of both parents, even if not always combined in equal proportions, and that no specimen morphologically identified as *O. mascula* or *O. pauciflora* possessed detectable rDNA of the other species. The majority of the hybrid specimens (12 individuals) showed a preponderance of *O. mascula* rDNA, three had approximately equal proportions of parental repeats and one had a preponderance of *O. pauciflora* rDNA. As the parental species have different amounts of rDNA, as inferred from dot blot hybridization, it has been deduced that the 12 specimens may represent F_1 or successive, presumably non-recombinant progenies, while the last four specimens may represent either F_2 genotypes or backcrosses with *O. pauciflora*.

Additional key words: dot blot, hybridization area, introgression, *Orchis* × *colemanii*.

Introduction

One of the tantalizing questions on orchid biology is why closely related species occur in sympatry without freely hybridizing, in spite of the apparently weak development of reproductive barriers to hybridization in the family.

The genetic isolation within genus *Orchis* seems to be based on specialized relationships with pollinators and elaborate systems which mechanically prevent the transfer of the pollinia to the stigmata of different species (Dafni 1987, Dressler 1981, Van der Pijl and Dodson 1966, Nilsson 1992). As a consequence, most of the hybridization in *Orchis* is restricted to few plants; this is an indication of the efficiency of the above mentioned isolation mechanisms, as all the members of the genus are said to be 'potentially interbreeding (Dafni 1987, Duperrex 1961).

However, documented evidence of widespread hybridization between sympatric species exists, and some hybrid swarms have been detected in orchids (*e.g.*, Arduino *et al.* 1996, Dafni and Ivri 1979). In these cases, not all the hybrids always show morphological intermediacy between parents, and some parental characters are expressed in full, at least in some individuals (Dafni and Ivri 1979). This is probably a consequence of the fact that morphological characters differentiating closely related species are often under simple genetic control (Rieseberg 1995, Hilu 1983, Gottlieb 1984). For these reasons, morphological observation alone cannot always correctly attribute an hybrid fenotype and, especially, cannot help distinguish introgressive events from F_1 hybrid variability or F_2 hybrid segregation. Molecular markers, as ribosomal DNA (rDNA), provide a powerful tool in investigating the occurrence and extent of hybridization and introgression (Doyle and Doyle 1988, Arnold *et al.* 1990,

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Rieseberg and Carney 1998). In fact, this marker typically possesses simple modes of expression and inheritance and may provide evidence of low-level introgression in a much more accurate fashion than morphological characters (Arnold *et al.* 1987). Usually hybridization and the formation of hybrid zones may result in a clinal variation in the pattern of parent-specific rDNA (*i.e.* the co-occurrence of rDNA of the other species in one or in both the parents) which is a consequence of backcrossing (Arnold *et al.* 1987). The observation of these clines, in terms of occurrence and slope has been often used as a definite clue for inferring introgression in plants (Nason *et al.* 1992).

O. mascula (L.) L. and *O. pauciflora* Ten. are two closely related species (Aceto *et al.* 1999), morphologically very similar and sharing the same habitat preferences and flowering time, which sometimes produce small hybrid swarms. The resulting hybrid is *O.* × *colemanii* Cortesi, described from Latium (Central Italy) at the beginning of the century (Cortesi 1907). This hybrid, also considered by some authors a hybridogenous species (Del Prete and Miceli 1981), is rather frequently found within mixed populations of *O. mascula* and *O. pauciflora* on the calcareous mountains of peninsular Italy (Nazzaro *et al.* 1991/1992, 1995). In this paper, we report the rDNA analysis of a small *O.* × *colemanii* population found in Southern Italy.

Materials and methods

A locality has been found near Sassano (National Park of Cilento, province of Salerno, Southern Italy), in which *O.* × *colemanii*, *O. mascula*, *O. pauciflora*, and other orchids (namely *O. morio* L., *O. papilionacea* L. subsp. *papilionacea* and *O. simia* Lam.) occur sympatrically. This population covers approximately 400 m^2, (1200 m a.s.l.) and occurs in a stony meadow, in the presence of scanty specimens of *Alnus cordata* (Loisel.) Desf., *Crataegus monogyna* Jacq., *Ostrya carpinifolia* Scop., and *Rosa* spp.

A total of 16 *O.* × *colemanii*, 15 *O. pauciflora* and 15 *O. mascula* flowering individuals were sampled in this area. An additional individual of *O. mascula* was sampled from Miralago (Matese Massif, province of Caserta, Southern Italy) and an individual of *O. pauciflora* was sampled from Moiano (Mount Faito, province of Napoli, Southern Italy). These two specimens came from two localities in which the latter two species did not grew sympatrically. Inflorescences were collected and pressed after removal of a cauline leaf for DNA analysis. Voucher specimens of the inflorescences of all the plants used in the present study are deposited at NAP.

Fresh cauline leaves (about 0.5 g) of individual plants were frozen in liquid nitrogen and ground into a fine powder. Total DNAs were then extracted according to Caputo *et al.* (1991). *Eco*RV digestions, agarose gel electrophoreses, Southern transfers and dot blot hybridizations were carried out following standard procedures. Southern filters were hybridized at 65 °C against a PCR-amplified digoxigenin-labeled *O.* × *colemanii* DNA ribosomal fragment (including 18S 3' terminus, internal transcribed spacer I, 5.8S, internal transcribed spacer II, 25S 5' terminus) obtained *via* PCR amplification according to Caputo *et al.* (1997). Dot blots containing scalar amounts of *O. mascula* and *O. pauciflora* total DNAs were hybridized against a PCR-amplified digoxigenin-labeled DNA ribosomal fragment using the same primers as in the previous experiment, prepared by mixing equal amounts (10 ng) of *O. mascula* and *O. pauciflora* DNA ribosomal fragments. Replicate dot blots were hybridized against a PCR-amplified digoxigenin-labeled chloroplast DNA *trn*L fragment, amplified from *O.* × *colemanii* according to Gielly and Taberlet (1996). Probes preparation, filter hybridization, signal detection and probe removal were carried out according to the manufacturer's instructions (PCR DIG probe synthesis Kit and DIG detection kit, *Boehringer*, Mannheim, Germany), relative amounts of DNA on autoradiograms were estimated by acquiring images with a scanner and analyzing them with the *Biomax* 10 image analysis software (*Kodak Digital Science*, *EDAS*, USA).

Results

The hybrid population was morphologically variable, in terms of habit, flower colour, outer tepal shape and spur size. In vast majority of the specimens, however, characters were intermediate between those of the parental species. A single hybrid individual had yellowish flower, obtuse outer tepals and a spur longer than the ovary, all typical characters of *O. pauciflora*.

The 18S-25S ribosomal gene repeats of *O. mascula* and *O. pauciflora* differ in the presence of a recognition site for the restriction enzyme *Eco*RV. This site is present in all the investigated specimens of *O. mascula*, giving two fragments, 6.7 and 3.0 kb long, respectively, and absent from all the specimens of *O. pauciflora*, which show a single fragment 9.7 kb long (Fig. 1). The

diagnostic nature of this *Eco*RV restriction site has been demonstrated by assaying *O. mascula* and *O. pauciflora* specimens which grew at a great distance from the area in study (Matese Massif and Mount Faito, respectively). The difference between these ribosomal repeat type provides an useful marker that allows to distinguish the repeats of these two taxa even when they are combined in a hybrid plant.

All the 16 individuals of *O.* × *colemanii* in study showed the presence of both the repeat types (*i.e.*, those of *O. mascula* and *O. pauciflora*); no segregation of parental rDNA was observed (*i.e.*, hybrid phenotypes with rDNA from one parent only). However, the parental repeats were not always combined in equal proportions (Fig. 1).

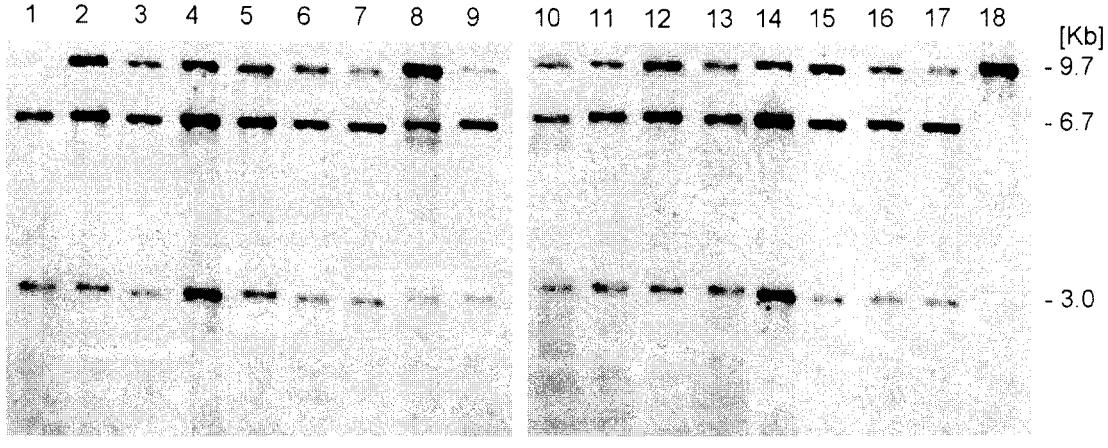

Fig. 1. Autoradiogram demonstrating the hybrid status of *O.* × *colemanii*. Total DNA was cut with *Eco*RV and hybridized against a PCR-amplified digoxigenin-labeled DNA ribosomal fragment. *Lane 1 - O. mascula, lanes 2 - 17 - O.* × *colemanii, lane 18 - O. pauciflora*. Numbers indicate lengths in kb.

According to the dot blot experiments (Fig. 2), the indagated samples of *O. mascula* contain approximately a double amount of rDNA genes as compared to *O. pauciflora*. The majority of our *O.* × *colemanii* specimens (12 individuals) have a preponderance of *O. mascula* rDNA (approximately 3:2 to 2:1), three have approximately equal proportions (1:1) of parental repeats (Fig. 1, *lanes 2,10,15*) and one has a preponderance (approximately 2:3) of *O. pauciflora* rDNA (Fig. 1, *lane 8*). This last sample was from the only individual among all the found hybrids with a preponderance of *O. pauciflora* morphological characters.

Therefore, all specimens morphologically referred to *O.* × *colemanii* revealed an additive rDNA pattern and no specimen morphologically identified as *O. mascula* or *O. pauciflora* possessed detectable rDNA of the other species.

Discussion

Ribosomal gene restriction profiles provided unambiguous evidence of hybridization in the zone of contact between the two species. All 16 individuals of *O.* × *colemanii* showed the combined repeat types of *O. mascula* and of *O. pauciflora* and no segregation was observed (Fig. 1). However the parental rDNA were not always combined in equal proportions: many individuals, in fact, had a preponderance of *O. mascula* repeat units. Different hypotheses may explain this ribosomal pattern in heterozygous individuals. Ribosomal DNA is inherited after the traditional Mendelian pattern (Saghai-Maroof *et al.* 1984) and therefore, if no gene conversion occurs, one would expect rDNA content variation simply in consequence of meiotic recombination in hybrids and segregation in F_2 (Arnold *et al.* 1987). As very little, if any, recombination within the rDNA locus is expected (Dvorák and Appels 1986) and rDNA genes are approximately twice more represented in *O. mascula* than in *O. pauciflora*, the majority of the hybrid specimens examined (*i.e.*, the 12 specimens with a *O. mascula*/ *O. pauciflora* rDNA ratio ranging from 2:1 to 3:2) are F_1 or successive, presumably non-recombinant progenies; the three hybrids with equal amounts of parental rDNA and the only hybrid with a preponderance of *O. pauciflora* rDNA may represent either F_2 genotypes or backcrosses with *O. pauciflora*. In the case of the latter specimen, we would tend for a backcross, as that individual was the only one conspicuously overloaded

with *O. pauciflora* characters.

The finding of a different number of rDNA copies in the two parental species is not unusual in plants (Arnold *et al.* 1990). In fact, differences in amounts of parental rDNA have been already observed in a previous study on the hybrid *O.* × *alata* (Caputo *et al.* 1997).

Given the different amount (Fig. 2) of copies of rDNA genes present in the two parental species (2:1), and if no gene conversion occurs, the presence of one individual with a preponderance of *O. pauciflora* rDNA rules out the hypothesis that rDNA is coded in a single locus. A minimum number of two loci, mapping on different chromosomes, would also account for the differences in parental rDNA ratio (from 2:1 to 3:2) found in the 12 above mentioned hybrids. As an alternative, unequal events of crossing over would account for the differences found in the genetic composition of the hybrids also in the case of a single locus.

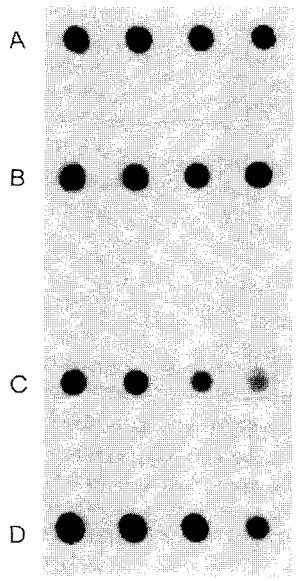

Fig. 2. Quantitative dot blot hybridization. From left to right: 75, 50, 25 and 12.5 ng of *O. pauciflora* (*row A* and *row C*), and of *O. mascula* (*row B* and *row D*). DNAs of *row A* and *row B* were hybridized against a PCR-amplified digoxigenin-labeled chloroplast DNA *trn*L fragment; DNAs of *row C* and *row D* were hybridized against a PCR-amplified digoxigenin-labelled DNA ribosomal fragment.

The occurrence of different rDNA types in hybrid plants may be easily maintained by vegetative reproduction or in the circumstance of F_1 population of low hybrid fertility; in fact, in the case of fully fertile and strictly inbreeding hybrids, homogenization of rDNA units *via* gene conversion or unequal crossing over would occur in few generations (Hillis *et al.* 1991, Wendel *et al.* 1995). As homogenization has not occurred in this case (probably as a consequence of the low reproductive fitness of the F_1 hybrids), the hypothesis of considering *O.* × *colemanii* as a species of hybridogenous origin, as reported by Del Prete and Miceli (1981), seems unlikely.

The complete absence of rDNA marker transfer from one parent species to the other, sometimes observed in sympatrically hybridizing plants, excludes that significant introgression occurs between *O. mascula* and *O. pauciflora* (Arnold *et al.* 1987). Our data would suggest that there is little or no gene flow between the two species. In fact, no plant attributed to one parental species on morphological grounds had rDNA repeats from the other species, even in the hybrid zone.

At present, therefore, with no evidence of significant introgression, either between the hybrids and the parents or between the parental species by themselves, *O.* × *colemanii* may only be regarded as a hybrid with little, if any, evolutionary potential.

Similar results have also been obtained by other authors in the study of other hybrid populations in *Orchis*. In a large isozyme study focusing on the hybridization zone between *O. laxiflora* and *O. palustris* (Arduino *et al.* 1996), hybrid populations were constituted mainly of F_1 types, with the other hybrid classes (F_2, backcrosses, introgressed) being represented in extremely low proportions. These authors hypothesized a strongly reduced hybrid fertility and a very limited mixing up of the two parental gene pools in the area of sympatry.

The karyological analysis of a hybrid swarm between *O. morio* and *O. papilionacea* (D'Emerico *et al.* 1996a) revealed that all examined hybrids were F_1 progeny, unfertile because of karyological barriers between parental taxa.

Allopolyploid speciation *via* hybrid formation is considered the main driving force in the evolution of *Orchidaceae*. However, allopolyploidy, well documented for the genera *Dactylorhiza* Necker, *Spiranthes* L.C.M. Richard and *Serapias* L. (Sun 1997, Hedren 1996a, Hedren 1996b, Arft and Ranker 1998), does not seem to be involved in *Orchis* speciation processes, which are said to be mainly allopatric (Dafni 1987). In fact, all the species of the latter genus possess a diploid karyotype 2n=36 or 2n=42, with the single exception of *O. papilionacea* with 2n=32 (Cauwet-Marc and Balayer 1984, D'Emerico *et al.* 1996b), and no polyploid numbers have been reported (with the possible exception of an unconfirmed 2n=80 for *O. patens*) (Sunderman and Von der Bank 1977). This would exclude the formation of polyploid species complexes as reported for *Dactylorhiza*.

Therefore, the formation of hybrids may simply represent a cost that *Orchis* species pay as a consequence of their peculiar reproductive biology. The majority of the species of the genus, including *O. mascula* and *O. pauciflora*, are nectarless species whose pollination is based either on visual/olfactory mimicry of nectariferous flowers or on casual visits by inexperienced bees

searching nectar in the spur. The latter is the case of *O. mascula*, which apparently does not mimic food-flowers, either in colour or in scent, but is pollinated by patrolling bees during their first flies (Nilsson 1983). This pollination mode assures a good out-breeding system without the burden of nectar production, but with a fraction of mistakes inherent to the process, at least in sympatric areas.

However, genetic or ethological barriers existing also between very closely related taxa, and despite their morphological similarities, (*i.e.*, the case of *O. mascula* and *O. pauciflora*) seem to prevent the occurrence of extensive hybridization and introgression: apparently the benefits of this pollination strategy in terms of energy saving minimize the loss deriving from the formation of hybrids (Dafni 1987).

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