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Autor: Aceto, Serena / Caputo, Paolo / Gaudio, Luciano

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Molecular approach to the identification and characterization of natural hybrids between *Orchis pauciflora* Ten. and *Orchis quadripunctata* Cyr. ex Ten. (Orchidaceae)

Serena Aceto[1], Paolo Caputo[2], Luciano Gaudio[1], Roberto Nazzaro[2] and Salvatore Cozzolino[3,4]

[1] Dip. di Genetica, Biologia generale e molecolare, Università di Napoli Federico II, Via Mezzocannone, 8, I-80134 Naples, Italy.
[2] Dip. di Biologia vegetale, Università di Napoli Federico II, Via Foria, 223, I-80139 Naples, Italy.
[3] Orto botanico, Università di Napoli Federico II, Via Foria, 223, I-80139 Naples, Italy.
[4] Author for correspondence

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Abstract

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Seven individuals of *Orchis* × *pseudoanatolica* Fleishm., a natural hybrid between *Orchis pauciflora* Ten. and *O. quadripunctata* Cyr. ex Ten. (*Orchidaceae*), collected in two close localities in Southern Italy, were characterized by employing molecular methods in order to identify their parents and to define their maternal lineages. Restriction analysis of PCR-amplified fragments containing the Internal Transcribed Spacer II (ITS2) of the nuclear ribosomal DNA showed that the plants are indeed hybrids between the two above mentioned species and allowed exclusion of *O. provincialis* Balbis, formerly indicated as potential parent, from the lineage. The chloroplast *trnL* (UAA) intron pattern of the hybrids indicates that *O. pauciflora* invariably provides the maternal line.

Key words: Hybridization; *Orchis* × *pseudoanatolica*; ITS; *trnL* intron

Introduction

Orchidaceae are an evolutionary young family, in which natural hybrids are widely diffused (Danesch and Danesch 1972; Ehrendorfer 1980; Hedrén 1996a,b). Often hybrids exhibit phenotypic intermediacy between the parental species and, therefore, are readily identifiable; however, the identification of hybrid individuals as such may be difficult when they resemble more one parent than the other or when the two parental species are very similar to each other (Steiner et al. 1994). Phenotypic intermediacy is often the result of interactions among complex genotypic traits, and can be only seldom associated with certainty to hybrid status (Gallez and Gottlieb 1982).

Difficulties in identification of orchid hybrids by using only morphological characters led some workers to explore the use of other sources of data, such as isozyme analysis (Steinbrück et al. 1986; Rossi et al. 1992, Rossi et al. 1995; Hedrén 1996a), karyology (Bianco et al. 1990; D'Emerico et al. 1996) and, more recently, DNA methods. The latter techniques have been widely used to identify plant hybrids and their maternal lineages (Rieseberg et al. 1990; Rieseberg and Brunsfield 1992; Kron et al. 1993). Recently, this approach has been applied also in the identification of orchid hybrids and the characterization of their parentage (Cozzolino and Aceto 1994; Caputo et al. 1997; Cozzolino et al. 1998).

In investigations dealing with molecular characterization of hybrids, two conditions must be met: 1. the putative parental taxa must be distinguishable from each other on the basis of their nuclear and chloroplast DNA (cpDNA); 2. the mode of cpDNA inheritance must be known.

Among the suitable DNA regions which can be used to detect parental taxa are the Internal Transcribed Spacers (ITS 1 or 2) of nuclear ribosomal DNA. Since nuclear markers are codominantly inherited, any hybrid accession must have the additive profile of the two parents, at least in F1 (Doyle and Doyle 1988; Rieseberg et al. 1990).

Separate indication of parentage (i.e., detecting maternal and/or paternal lineages), however, cannot be pursued by the way indicated above, as it requires uniparentally inherited markers; plastid genomes are strictly matrilinear in orchids (Corriveau and Coleman 1988; Harris and Ingram 1992), and therefore qualify as designators for maternal lineages. Among all the regions of cpDNA which can be employed to detect female parents, the *trnL* (UAA) intron (Taberlet et al. 1991) has been identified in all major green plant taxa.

The choice of ITS and *trnL* (UAA) intron depends upon various issues: first of all, both regions seems to have the appropriate range of variation to meet the purpose of detecting differences between close species (Baldwin et. al. 1995; Arnold et al. 1992; Watano et al. 1996; Vogel et al. 1998); moreover, they are comparatively short and, being included in nuclear ribosomal and chloroplast DNAs respectively, are present in large numbers of copies in each cell and represent an abundant fraction of the total genome. These characteristics make PCR amplification and RFLP analysis easy.

In this paper, molecular methods were used to confirm the hybrid status and to detect parentage of seven hybrid specimens identified as *Orchis* × *pseudoanatolica* Fleishm., a natural hybrid between *O. pauciflora* Ten. and *O. quadripunctata* Cyr. ex Ten., which early literature indications (Fleischmann 1914) reported as a hybrid between *O. provincialis* Balbis and *O. quadripunctata*.

The investigation was carried out by comparing restriction fragment profiles of the hybrids with those of the species which have been regarded as involved in parentage on morphological grounds and according to literature.

Historical background

A putative hybrid between *O. provincialis* and *O. quadripunctata* was found by Fleischmann (1914) in Curzola Island (Slovenja) and described as *O.* × *pseudoanatolica*. Later, J. Rholena described *O.* × *celakovskyi* Rholena, a hybrid between *O. pauciflora* and *O. quadripunctata*, found in Cetinje (Montenegro) (Rholena 1922).

While Camus and Camus (1928) reported *O.* × *pseudoanatolica* and *O.* × *celakovskyi* as different hybrids, Soó (1929) observed that in Curzola island only *O. pauciflora*, and never *O. provincialis*, had been reported. For this reason the parentage of *O.* × *pseudoanatolica* had to be reconsidered. On these grounds, the first description of a hybrid *O. pauciflora* × *O. quad-*

ripunctata must be attributed to Fleischmann (1914); later, in fact, Keller et al. (1930–40) synonymised *O.* × *celakovskyi* with *O.* × *pseudoanatolica*.

Recently, Wildhaber (1970) described *O.* × *buelii* Wildhaber as an hybrid between *O. provincialis* and *O. quadripunctata*. The only Italian record of *O.* × *pseudoanatolica* before the finding reported below was from Monte Sant'Angelo (Gargano, Apulia region) (Lorenz and Gembardt 1987).

Materials and methods

Plant material

Seven hybrid individuals were collected at flowering time in the mountains W of Vallo di Diano (province of Salerno, Southern Italy) in the spring of 1996 (four specimens) and 1997 (three specimens). The plants belonged to two different orchid populations, approx. 7 Km apart from each other. Various orchid species grew sympatrically in these two localities: *Aceras anthropophorum* (L.) R. Br., *Anacamptis pyramidalis* (L.) L. C. M. Richard, *Cephalanthera longifolia* (L.) Fritsch, *C. rubra* (L.) L. C. M. Richard, *Dactylorhiza latifolia* (L.) H. Baumann and Künkele, *D. saccifera* (Brongn.) Soó, *Gymnadenia conopsea* (L.) R. Br., *O. italica* Poiret, *O. mascula* (L.) L. subsp. *mascula*, *O. papilionacea* L. subsp. *papilionacea*, *O. pauciflora* Ten., *O. provincialis* Balbis, *O. purpurea* Hudson, *O. quadripunctata* Cyr. ex Ten., *O. simia* Lam., *Serapias vomeracea* (Burm.) Briq. subsp. *longipetala* (Ten.) H. Baumann and Künkele (Nazzaro et al. 1996). Single specimens of the putative parental species *O. pauciflora, O. provincialis*, and *O. quadripunctata* were collected from the first of the two localities. Voucher specimens of all the examined plants are deposited at NAP.

Methods

Total DNAs of the seven hybrid specimens and of the specimens of the putative parental species were extracted according to Caputo et al. (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′-CCAAACAACCCGACTCGTAGACAGC-3′) rDNA genes, respectively (Aceto et al. 1999).

Amplification of *trnL* (UAA) intron was carried out using the universal primers described by Taberlet et al. (1991). All PCR reactions (100 µl final volume) were conducted in a thermocycler (Perkin Elmer Cetus 9600) for 30 cycles. Initial conditions were as follows: 30 sec denaturation at 94 °C, 1 min annealing at 55 °C, 45 sec extension at 72 °C. Samples were denatured for 9 min at 94 °C before the beginning of the first cycle as requested by the manifacturer for the activation of *Taq* Gold (Perkin Elmer); extension time was increased of 3 sec/cycle; extension was further prolonged for 7 min at the end of the last cycle.

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 µl TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) were carried out by centrifugation at 7000g for 6 min to eliminate any salt residue.

The restriction enzymes for ITS2 and *trnL* intron were chosen according to the results of a computer-aided restriction analysis of published (Aceto et. al. 1999 and related Gene Bank accessions) and unpublished (Aceto et al., unpublished; Widmer et al., pers. comm.) sequences of all the sympatrically growing orchid species (Nazzaro et al. 1996). For ITS2 only the three restriction enzymes yielding profiles autapomorphic of each potential parent (*Bgl*II,

*Stu*I, *Sma*I) were chosen. Purified fragments were then digested with the restriction endonucleases *Bgl*II, *Stu*I, *Sma*I (ITS2) and *Hpa*II (*trn*L intron), electrophoretically separated on a 2% agarose gel, stained with ethidium bromide and photographed on a UV transilluminator. A 100 base pair (bp) ladder (Pharmacia Biotech) was used as molecular weight marker.

The purified fragments from all the hybrid specimens were double-strand sequenced in both directions by using a modification of the Sanger dideoxy method (Sanger et al. 1977) as implemented in a double strand DNA cycle sequencing system with fluorescent dyes and using the same primers as in Aceto et al. (1999). Sequence reactions were then loaded into a 373A Applied Biosystems Automated DNA sequencer (Applied Biosystems, Foster City, CA, U.S.).

Results

The seven plants in study were 16–30 cm high, with a stem purple in the uppermost part (as in *O. quadripunctata*), with 5–8 mucronate, spotless leaves, crowded in a basal rosette. Bracts were purplish, lanceolate-acuminate, uninervate, long about 4/5 of the ovary. Inflorescences were cylindrical (as in *O. quadripunctata*), crowded, with 11–18 flowers. Flowers were purple-violet (as in *O. quadripunctata*). Outer lateral tepals were ovate and patent, and some of them with a reflexed apex (as in *O. pauciflora* and *O. provincialis*). Labellum was purple, trilobate, wider than long (1.2 × 0.8 cm), with dark purplish spots (as in *O. quadripunctata*) on yellow background (as in *O. pauciflora* and *O. provincialis*) and crenulate borders (as in *O. pauciflora* and *O. provincialis*). The median lobe showed two lobules separated by a tooth (as in *O. pauciflora* and *O. provincialis*) (Fig. 1). The flowers of all seven examined hybrids show mature pollinia with a viscous essudate. The observation of both the ITS2-containing and the *trnL* (UAA) intron fragments has shown no length differences between the three parental species in the amplified regions.

The ITS2-containing fragments obtained from *O. quadripunctata, O. pauciflora* and *O. provincialis* were approx. 600 bp in length, while raw *trnL* (UAA) fragments were approx. 580 bp in length.

The length of the amplified fragments was identical in the potential parents (as demonstrated in Aceto et al. 1999); therefore a restriction analysis was carried out. For ITS2, the selection of restriction enzymes was aimed at choosing sites which were different between parents and unique to them as compared to all the other sympatrically growing orchids. Parental ITS2-containing fragments digested with *Bgl*II showed a single restriction site in *O. pauciflora* (with fragments approx. 470 bp and 130 bp long) and no site in *O. quadripunctata* and *O. provincialis*. The ITS2-containing fragments of all the hybrids, when digested, showed the presence of both an undigested fragment (as in *O. quadripunctata* and *O. provincialis)* and the *Bgl*II restriction site typical of *O. pauciflora* (Fig. 2a).

In the same way, a *Stu*I restriction site was present only in the ITS2-containing fragment of *O. quadripunctata* (with fragments approx. 470 bp and 130 bp long) and absent in *O. provicialis* and *O. pauciflora*. This site was observed, together with an undigested fragment, also in all the examined hybrid ITS2-containing fragments (Fig. 2b). Finally, a *Sma*I restriction site, heterozygously present in *O. provincialis* (with fragments approx. 310 bp and 290 bp long), has not been detected in any hybrid (Fig. 2c).

As far as cpDNA is concerned, the *trnL* (UAA) intron of *O. quadripunctata* digested with *Hpa*II showed two fragments (440 bp and 140 bp), while no site was detected in either *O. pauciflora* or the hybrids (Fig. 2d).

The ITS2 sequence of all investigated hybrids corresponded to the one expected for a F1 hybrid between *O. quadripunctata* and *O. pauciflora*, with paralogies in the positions in which

Fig. 1. A specimen of *Orchis* × *pseudoanatolica.*

the two parental species are different in sequence. At the only two positions in which the parental species share nucleotides different from those in the corresponding positions of *O. provincialis*, the hybrid sequence was identical to those of *O. quadripunctata* and *O. pauciflora,* and no paralogues were present.

Discussion

The parentage of *O.* × *pseudoanatolica* has been controversial for the past (Fleischmann 1914, Rholena 1922; Camus and Camus 1928, Soó 1929), especially as a consequence of the strong resemblance between two of the three species which have been regarded along

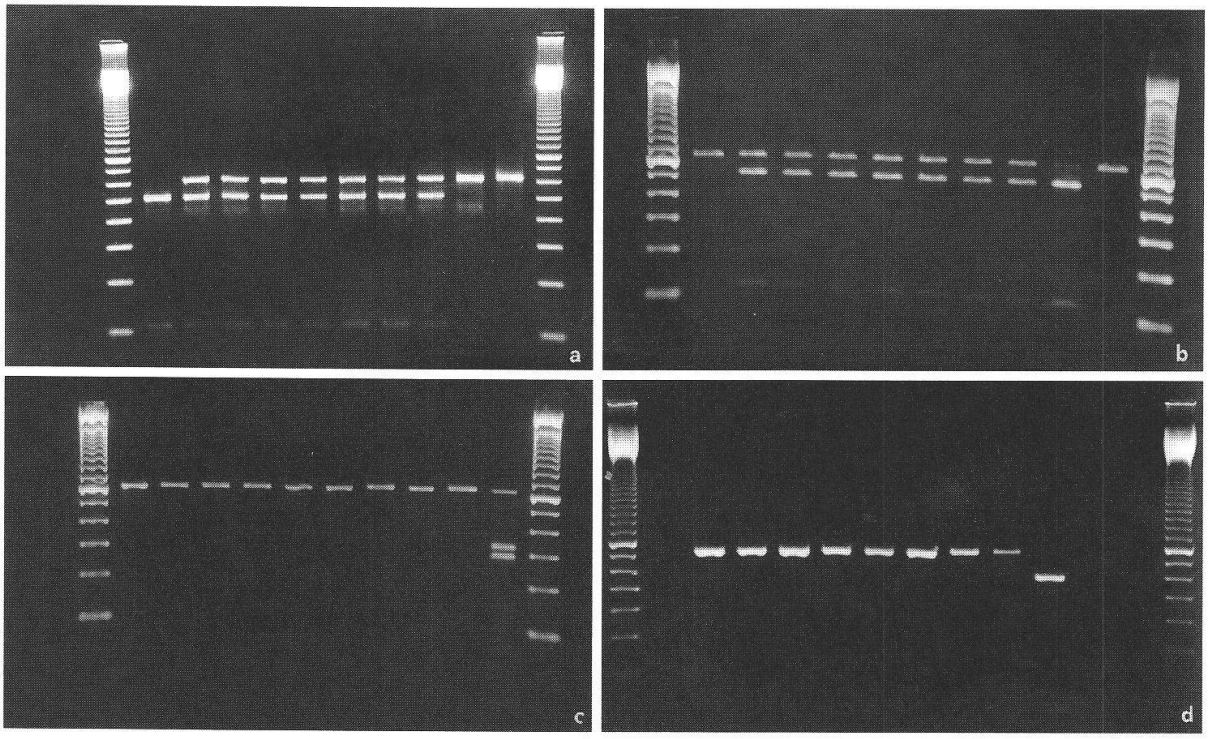

Fig. 2. a) Gel electrophoresis of ITS2 *Bgl*II digestion of *Orchis pauciflora* (line 2), seven specimens of *O.* × *pseudoanatolica* (from line 3 to line 9), *O. quadripunctata* (line 10), *O. provincialis* (line 11), and molecular 100 bp ladder (line 1 and line 12). b) Gel electrophoresis of ITS2 *Stu*II digestion of *O. pauciflora* (line 2), seven specimens of *O.* × *pseudoanatolica* (from line 3 to line 9), *O. quadripunctata* (line 10), *O. provincialis* (line 11), and molecular 100 bp ladder (line 1 and line 12). c) Gel electrophoresis of ITS2 *Sma*I digestion of *O. pauciflora* (line 2), seven specimens of *O.* × *pseudoanatolica* (from line 3 to line 9), *O. quadripunctata* (line 10), *O. provincialis* (line 11), and molecular 100 bp ladder (line 1 and line 12). d) Gel electrophoresis of trnL intron *Hpa*II digestion of *O. pauciflora* (line 2), seven specimens of *O.* × *pseudoanatolica* (from line 3 to line 9), *O. quadripunctata* (line 10), and molecular 100 bp ladder (line 1 and line 11).

time as involved in the cross, *O. pauciflora* and *O. provincialis*. The recent description of *O.* × *buelii*, a hybrid between *O. provincialis* and *O. quadripunctata* (Wildhaber 1970), contributed to further complicate assessment of the ancestral line of *O.* × *pseudoanatolica,* which shows great morphological similarity with *O.* × *buelii*: the main difference between the two hybrids is the presence of dark spots on the leaves of the latter (this is also one of the main discriminating characters between *O. provincialis* and *O. pauciflora*); such characters are not necessarily transferred to all progeny (see Halácsy (1881) for a similar case of a hybrid between *Dactylorhiza latifolia* (L.) H. Baumann and Künkele and *D. maculata* (L.) Soó, in which no hybrid plants did have dark spots), and therefore the presence of a spotless progeny from a cross between *O. provincialis* and *O. quadripunctata* (i.e., of a spotless *O.* × *buelii*) cannot be ruled out in principle. No molecular evidence is available about the parentage of *O.* × *buelii*. However, the results shown here indicate that *O. provincialis* is not involved in the lineage of the hybrids investigated in our study.

ITS restriction patterns of the hybrids, in which exclusive parental bands are always present, clearly indicate that *O. quadripunctata* and *O. pauciflora* provide parental lineages (as inferred by morphology) and at the same time rule out *O. provincialis* as potential parent.

In fact the presence of a *Bgl*II site of *O. pauciflora* and of a *Stu*I site of *O. quadripunctata* has been detected in all the investigated hybrids, while the *Sma*I restriction site of *O. provincialis* has never been detected (Fig. 2a, 2b, 2c).

All hybrid plants possess the exclusive sites of *O. pauciflora* and of *O. quadripunctata* and both sites have not been detected in any other sympatric *Orchis* or related genera present in the area.

An interesting issue which may be addressed here is that these unique sites do not seem to be present in all the copies of the parental ITS's. In fact, a small fraction of the amplification products remains undigested (Fig. 2b, 2c). This indicates an incomplete identity among the ribosomal sequences within single individuals, which may be attributed to recent gene flow. Regardless, we decided to use only one specimen of each of the potential parents, because repeated sequencing experiments carried out on individuals of the same species from different localities (Aceto et al., unpublished; Widmer et al., unpublished) showed that ITS sequence is (or at least, the great majority of the copies of ITS are) invariant in all the species of *Orchis* and related genera tested.

The presence of mature pollinia on all the hybrid flowers (even in absence of direct evidence of viability) suggests that *O.* × *pseudoanatolica* may perhaps play a role (clearly limited by its rarity) in the gene flow between parental species (see the undigested *O. quadripunctata* rDNA fragment in Fig. 2b). The ITS sequences of the investigated hybrids also confirmed their parental lineage and allowed the exclusion of *O. provincialis* from the parentage. However, the presence of a single base deletion (5′–3′ position 186) in the ITS2 of *O. quadripunctata* as compared to that of *O. pauciflora* did not allow legibility of the hybrid sequence beyond that position in either direction, so preventing the double check which is standard procedure in DNA sequencing. Moreover, the dye-terminator protocol which was used in our automated double-strand PCR-mediated sequence analysis gives signals of different intensity for different bases (particularly, it displays a negative bias towards G's); therefore, an assessment of the percentage of contribution of the parental genomes to the hybrid formation was impossible. A more reliable approach in this case would consist in cloning the PCR fragments into plasmid vectors and using the dye primer technique (Perkin-Elmer, USA); however, in this case obtaining the sequence for many clones would be necessary, and this may render this procedure not effortless. For these reasons, we regard the restriction approach used here as an ideal tool, at least in term of cost/effectiveness, for F1 hybrid recognition.

After exclusion of *O. provincialis* from the parental lines, the differences in the cpDNA restriction patterns between *O. quadripunctata* and *O. pauciflora* enabled us to establish the maternal lineage of our *O.* × *pseudoanatolica* specimens. In fact, restriction analysis of the *trnL* (UAA) intron showed that the fragment pattern of the hybrids invariably corresponds to that of *O. pauciflora*.

As parental lineages had already been deduced by the restriction analysis of ITS2, it was not necessary, in the present circumstance, to find autapomorphic sites in parental species, as the only requirement was that sites were not shared by both parents.

Identification of maternal lineages is especially useful for understanding reproductive mechanisms in parental species and for providing information on the direction of gene flow in the formation of hybrids. The data shown here, even if not statistically significant, lead us to suppose that in the case of *O. quadripunctata* and *O. pauciflora* pollination is unidirectional. Presently, we are not able to ascertain whether the two separate hybrid findings originate from a single event (even if the space separating the two findings would suggest independent occurrences). Unidirectionality of gene flow may be attributed to the presence of reproductive barriers in one of the two parents. Both species may be non-model mimic of nec-

tar-rewarding plants (Dafni 1987) but pollination biology studies on the two species are still lacking (Van der Cingel 1995). In order to assess statistical significance for the hypothesis of unidirectionality, and potential introgression, which seems to be one of the chief speciation mechanisms in orchids (Ehrendorfer 1980; Hedrén 1996b), further analysis will be carried out as soon as other of these rare hybrids will be available.

Finally it is to be noted that PCR-mediated approaches to hybrid identification allow the use of extremely small amounts of DNA and, accordingly, of very small amounts of leaf tissue for DNA extraction. This technique, therefore, may be especially useful when field hybridization studies need to be carried out on rare and endangered plants.

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