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Autor: Pellegrino, Giuseppe / Cozzolino, Salvatore / D'Emerico, Saverio

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The taxonomic position of the controversial taxon *Orchis clandestina* (Orchidaceae): Karyomorphological and molecular analyses

Giuseppe Pellegrino[1], Salvatore Cozzolino[2], Saverio D'Emerico[3] and Paolo Grünanger[4]

[1] Dipartimento di Ecologia, Università della Calabria, I-87036 Arcavacata di Rende, CS (Italy)
[2] Orto Botanico, Università di Napoli Federico II, Via Foria 223, I-80139 Napoli (Italy)
[3] Dipartimento di Biologia e Patologia Vegetale, Università di Bari, Via Orabona 4, I-70125 Bari (Italy)
[4] Dipartimento di Chimica Organica, Università di Pavia, Via Taramelli 10, I-27100 Pavia (Italy)

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Abstract

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The controversial taxon *Orchis clandestina* Hautz., a micro-endemic entity of the Ligurian coast (province of Genoa), was investigated with the aim to define its taxonomic position. Morphological characters resulted to be intermediate between *O. patens* and the *O. mascula – O. provincialis* group. Both, karyomorphological (C-banding) and molecular (rDNA, ITS sequences) analyses, indicate a hybrid origin of *O. clandestina* and allowed us to recognize *O. patens* and *O. provincialis* as parental species, thus ruling out any contribution of *O. mascula* in the hybrid formation. In view of the present results we assign a hybrid status to *O. clandestina* and synonymize it with *Orchis x fallax* (de Not.) Willk. & Lg.

Key words: Karyomorphology, rDNA analysis, ITS, hybridization.

Introduction

Due to the frequent occurrence of hybridization events in Euro-Asiatic orchids, new morphological combinations may easily arise. As a consequence difficulties in identification of orchid hybrids emerge if these do not exhibit phenotypic intermediacy between the parental species, an erroneous taxonomic status may then be attributed to these findings.

Hautzinger (1978) described *Orchis clandestina* Hautz. as a new species, endemic to a limited area, east of the city of Genoa (Italy). The recognition as a distinct species, clearly related to *O. patens* Desf., and therefore belonging to the same section, was essentially based on morphological observations and on the reported chromosome number of 2 n = ±42 that was different from *O. patens* (2 n = ±80). Subsequently, *O. clandestina* has been treated controversially in the orchidological literature: it was not mentioned in Buttler (1986), in Baumann and Künkele (1982, 1988) and in Delforge and Tyteca (1984), whereas it was referred in the monograph by del Prete and Tosi (1988). Liverani (1991) initially hypothesized for this taxon a hybrid origin

between *O. patens* and *O. provincialis*, while Delforge (1994) renamed the taxon *O. ligustica* Ruppert (pro hybr.), thus implying a hybrid origin between *O. patens* and *O. mascula*.

Two hybrids of *O. patens*, either with *O. mascula* L. or with *O. provincialis* Lam. & Dc., have already been reported from the same geographic area: the former was described by Ruppert (1933) as *O.* × *ligustica*, the latter was originally described by de Notaris (1844) as *O. brevicornu* var. *fallax* and by Camus (1928) as *Orchis x subpatens* and finally named *Orchis* × *fallax* (De Not.) Willk. & Lg. by Hautzinger (1978).

In the present paper, we carried out a karyomorphological and a molecular analysis, combined with morphological observations, with the aim to clarify the taxonomic position of *O. clandestina* and its relationships with *O. patens, O. provincialis* and *O. mascula* which were considered to be involved in its origin.

Materials and methods

Plant materials of *O. clandestina*, *O. patens*, *O. provincialis* and *O. mascula* were collected in 1996 and 1998 in Sorlana (Genoa), close to the *locus classicus* of *O. clandestina* (S. Giulia, Genoa, Ligurian region) from which *O. clandestina* has disappeared by now.

Mitotic chromosomes were observed in tissues of immature ovaries. For C-banding, the ovaries were pre-treated with 0.3% aqueous colchicine at room temperature for 2 hr, then fixed in ethanol-glacial acetic acid (3:1 v/v) and stored at –20°C for one day to several months. Subsequently, ovaries were squashed in 45% acetic acid; coverlips were removed by the dry ice method (D'Emerico et al. 1996) and the preparations air-dried overnight. Slides were then immersed in 0.2 n HCl at 60 °C for 3 min, thoroughly rinsed in distilled water and then treated with 4% $Ba(OH)_2$ at 20 °C for 4–5 min. After very thorough rinsing they were incubated in 2 × SSC at 60 °C for 1 hr, and stained in 3–4% Giemsa (BDH) at pH 7 according to D'Emerico et al. (1996).

For molecular analysis, fresh cauline leaves (approx. 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).

PCR reaction: the Internal Transcribed Ribosomal Spacers (ITS I and ITS II) were amplified by Polymerase Chain Reaction (PCR), using two pairs 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′), in the 3′ region of the 5.8S (5′-TTGCAGAATCCCGTGAACCATCG-3′) and in the 5′ region of the 25S (5′-CCAAACAACCCGACTCGTGACAGC-3′) rDNA genes. All PCR reactions, with 10 ng of DNA as template, (100 µl final volume) were carried out in a thermal cycle (Perkin Elmer 2600) 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; extension time was increased by 3 sec/cycle; extension was further prolonged for 7 min at the end of the last cycle. Amplified fragments were purified using Microcon 100 microconcentrators (Amicon MWCO 100,000). Purified PCR products were digested with the restriction endonucleases *Alu* I (for ITS I) and *Pvu* II (for ITS II), electrophoretically separated on a 3% agarose gel (Metaphore agarose FMC), stained with ethidium bromide and photographed on a UV transilluminator. A 100 base pair (bp) ladder (Pharmacia Biotech) was used as a molecular weight marker. These enzymes were chosen according to the results of a computer-aided restriction analysis of the published sequences (Aceto et al. 1999 and related Gene Bank accessions) of all the sympatrically growing orchid taxa. This analysis showed that these restriction sites are exclusive of *O. mascula* or *O. provincialis* and *O. patens*, respectively.

RFLP analysis: 100ng of DNAs of all examined samples were digested with a variety of restriction endonucleases, namely *Bam*HI, *Eco*RI, *Eco*RV and *Hind*III. Endonucleases digestions, agarose gel electrophoreses, Southern transfers, and hybridization were carried out following standard procedures as described in Sambrook et al. (1989). Southern filters were hybridized at 65°C against a PCR-amplified digoxigenin-labelled *O. mascula* DNA ribosomal fragment (ITS I + 5.8S + ITS II) obtained via PCR amplification according to Pellegrino et al. (2000). Probe preparation, filter hybridization, signal detection and probe removal were carried out according the suppliers recommendations (PCR DIG probe synthesis kit and DIG detection kit, Roche-Boehringer Mannheim).

Results

Morphological and field observation

Some external features, especially the green coloration of inside surface of the lateral sepals, justify the placement of *O. clandestina* in the *O. patens* group, but other morphological characters appear intermediate between the *O. patens* group and the *O. mascula – O. provincialis* group. Table 1 shows some selected typical morphological features of the four taxa. Both *O. mascula* and *O. provincialis* are widely distributed in the same regions where *O. patens* and *O. clandestina* grow sympatrically, i.e. along the Ligurian coastal region east of Genoa. *O. clandestina* seems to have suffered from a pronounced decrease in its occurrence during the last decade, as revealed by its total disappearance in its "*locus classicus*" (Santa Giulia, Genoa). Interestingly, no significant blooming shift in comparison with *O. patens* was observed, in disagreement with Hautzinger's observation of a 15-day time lag.

Karyological analysis

The karyological analysis, carried out on several individuals, all morphologically corresponding to the original description of *O. clandestina*, revealed in all accessions a chromosome number of 2n = 63. This value differs from those (2n = ±42) reported by Hautzinger (1978). The reported chromosome number 2n = 63 indicates a potential hybrid origin of the investigated taxon, suggesting *O. patens* (the only tetraploid species in the genus *Orchis*) as one of the two parental species. Indeed, all individuals of *O. patens* showed a chromosome number 2n = 84, which is in agreement with the value reported by Hautzinger (2n = ±80). Both *O. mascula* (Scrugli et al. 1976) and *O. provincialis* have chromosome numbers 2n = 42 (Scrug-

Table 1. Synopsis of the main discriminating features between *Orchis clandestina* and its putative parents.

Feature	*O. patens*	*O. clandestina*	*O. mascula*	*O. provincialis*
Stem	Purplish at least in the upper part	Usually green up to top	Usually violet-purplish spread	Green
Basal leaves	Usually unspotted	With dark brown spots	Unspotted or with large purplish spots	With large purplish spots
Spike	Fairly lax	Fairly lax	Fairly dense	Lax to dense
Lateral sepals	Inside a green blotch with pink spots and pink edges	Inside pink, green-soffused, with pink spots	Concoloured to the lip	Inside whitish
Flower colour	Pink	Purplish	Pink to reddish	Yellow
Lip	3-Lobed, central part whitish with purple spots	3-Lobed, central part whitish or yellowish with purple spots	3-Lobed, central part whitish with purple spots	3-Lobed, central part orange-yellow with purple spots
Lip's lateral lobes	Relatively narrow	Relatively large	Large	Relatively large
Spur	Thick, conical, short (length < 1/2 ovary)	Thick, subcilindrical, rather long (length 1/2–3/4 ovary)	Cilindrical, long (length · ovary)	Cilindrical, long (length · ovary)

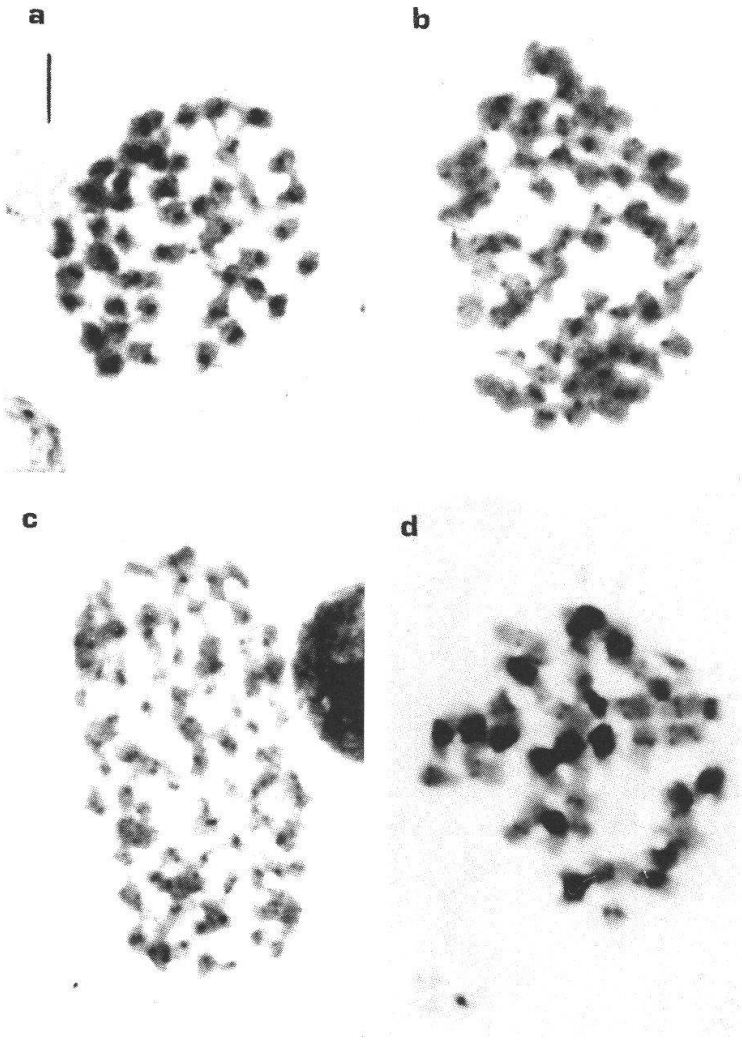

Fig. 1. Giemsa C-banded metaphases from tissues of immature ovaries of a) *Orchis provincialis*, 2n = 42; b) *O. clandestina*, 2n= 63; c) *O. patens*, 2n = 84 and d) *O. mascula*, 2n= 42.

li 1977) and this number has been also found in all analysed specimens from the Ligurian localities. C-Banded metaphase chromosomes of *O. patens* and *O. provincialis* show similar heterochromatin bands organization. In fact, they prevalently possess small centromeric bands, the heterochromatin being observed as two dots localized in the primary constrictions. However, in *O. provincialis* some chromosomes present also a telomeric heterochromatin.

In contrast with *O. patens* and *O. provincialis,* the banding pattern of *O. mascula* is clearly distinct for the presence of numerous chromosomes characterized by large heterochromatic bands (Fig. 1).

The banding patterns of examined accessions of *O. clandestina* show a comparable organization of heterochromatin bands to that of *O. patens* and *O. provincialis* while no chromosome displayed large heterochromatic centromeric bands as observed in *O. mascula.*

Molecular analysis

From the molecular study it appears that the ITS-containing fragments obtained from the four taxa were approximately 380 (ITS I) and 600 (ITS II) bp in length.

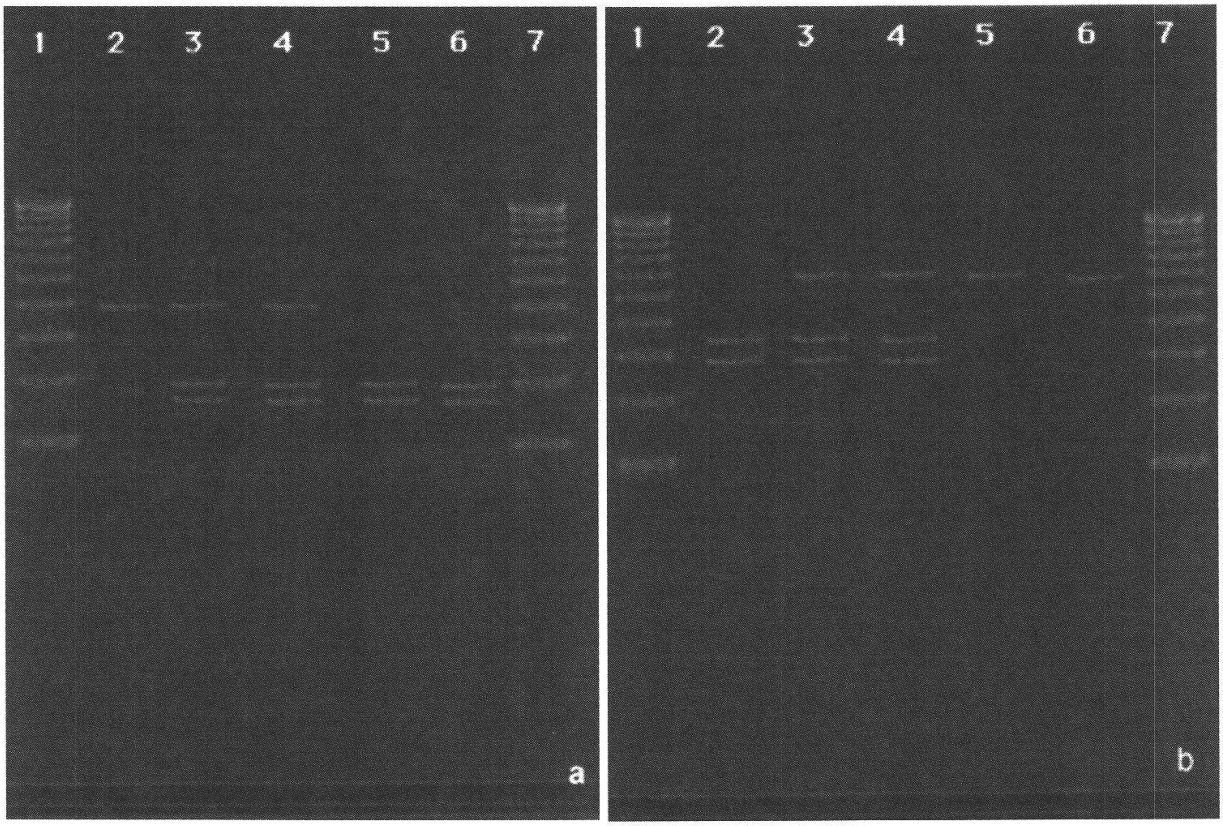

Fig. 2. a) Gel electrophoresis of ITS I: *Alu* I digestions of *O. patens* (line 2), two specimens of *O. clandestina* (lines 3 and 4), *O. mascula* (line 5), *O. provincialis* (line 6), and molecular 100 bp ladder (line 1 and line 7). b) Gel electrophoresis of ITS II: *Pvu* II digestions of *O. patens* (line 2), two specimens of *O. clandestina* (lines 3 and 4), *O. mascula* (line 5), *O. provincialis* (line 6), and molecular 100 bp ladder (line 1 and line 7).

ITS I-containing fragments digested with *Alu* I showed a single restriction site in *O. mascula*, *O. provincialis* and *O. clandestina* (with two fragments approx. 200 bp and 180 bp long) and no site in *O. patens* (Fig. 2a). The ITS II-containing fragments digested with *Pvu* II showed a single restriction site in *O. patens* and *O. clandestina* (with two fragments approx. 280 bp and 320 bp long) and no site in *O. mascula* and *O. provincialis* (Fig. 2b).

To distinguish between *O. mascula* and *O. provincialis*, which possess the same digestion pattern, a restriction fragment length polymorphism (RFLP) analysis of nuclear rDNA was carried out. The ribosomal gene repeats of *O. mascula* and *O. provincialis* differ in the presence of an additive site for the restriction enzyme *Eco*RV in *O. mascula*, giving two hybridization fragments, 6.7 and 3.0 kb long respectively. This additional site is absent in *O. provincialis*, which shows a single hybridization fragment 9.7 kb long. All *O. clandestina* accessions showed a single hybridization fragment 9.7 kb long as *O. provincialis*, with no evidence of the additive *Eco*RV site (Fig. 3).

Discussion

An approach of combining karyological and molecular data represents a powerful methodology to correctly identify the taxonomic position of controversial taxa. These techniques

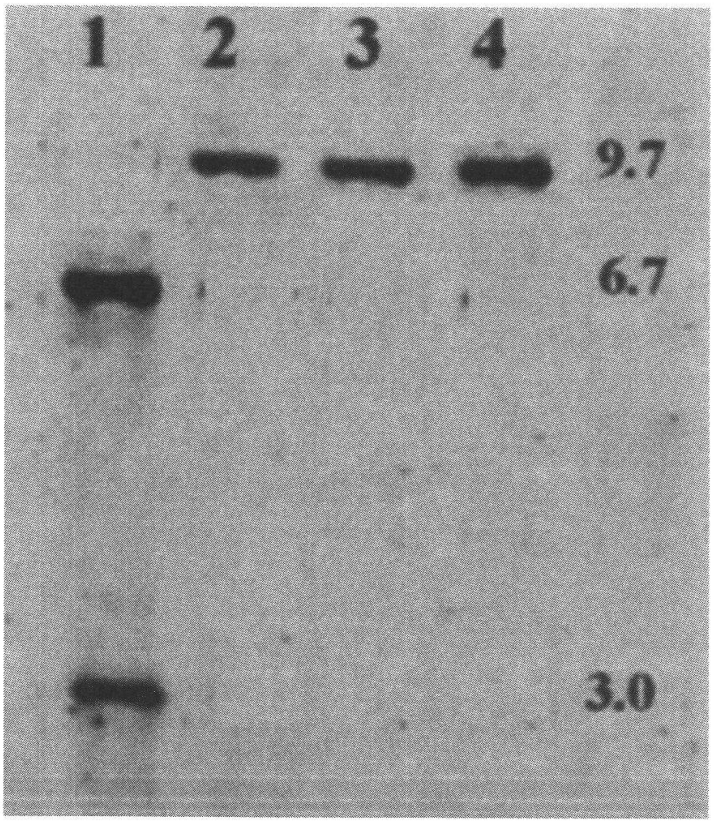

Fig 3. Total DNAs digested with *Eco*RV and hybridized against a PCR-amplified digoxigenin-labelled DNA ribosomal fragment. Lane 1) *O. mascula*; 2 and 3) *O. clandestina*; 4) *O. provincialis*. Numbers indicate length in kb.

need a very small amount of plant material and thus prove to be especially useful when studies on rare and endangered plants, as in the case of the species in the present work, need to be carried out.

Even if the morphological observations may suggest a potential hybrid origin for *O. clandestina*, morphology alone does not always allow a clear recognition of a hybrid status and especially does not always grant the correct identification of its parental lineages when closely related taxa are involved.

The only observation of chromosomal number of *O. clandestina* suggests a hybrid origin with one of the parental species being *O. patens*, the only *Orchis* species with 2n=84. However, chromosomal number alone does not help in recognizing the other species involved in *O. clandestina* origin. The C-banding analyses, which allow to distinguish among complements with same chromosome numbers (2n=42), indicate in *O. provincialis* the other putative parental species. In fact, the banding pattern of *O. mascula* is clearly distinct by the presence of large heterochromatic centromeric bands. The absence of this pattern in the *O. clandestina* chromosomal plates, therefore, excludes *O. mascula* as a putative parental species.

The presence of ITS sequences from different species in *O. clandestina* showed that it has indeed a hybrid origin. Furthermore, visually the hybrid pattern shows equally amplified amounts of the ribosomal DNAs of the two parents. The absence of any predominant DNA pattern allows us to conclude that *O. clandestina* could represent F1 progeny or at most the result of a recombination between two hybrid specimens. In fact, if a back-cross occurs, the ratio between parental DNA in the hybrids is shifted in favour of one of the parental species.

Moreover, as far as the parental species are concerned, from restriction pattern of amplified ITS, it follows that one parental species is *O. patens* while the other one may be either *O. mascula* or *O. provincialis*. However, the RFLP analysis rules out *O. mascula* as parental species due to the absence of *O. mascula* ribosomal DNA in all hybrid accessions.

In conclusion, the results of both karyomorphological and molecular (DNA) analyses clearly show that the *O. clandestina* is indeed a hybrid taxon, with *O. provincialis* and *O. patens* being the parental species. *O. clandestina* should hence be considered a synonym *of Orchis* × *fallax* (De Not) Willk. & Lg.

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