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# Morphological and molecular characterization of *Aceras anthropophorum* x *Orchis simia* hybrids

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***Abstract.*** Five specimens of a putative natural hybrid between *Aceras anthropophorum* and *Orchis simia*, formerly known as x*Orchiaceras bergonii* (Nanteuil) E. G. Cam., were characterized both on morphological and molecular grounds in order to confirm their hybrid status. The morphological characters of the specimens showed intermediacy between those of the parental species, and ITS restriction patterns of the nuclear ribosomal DNA confirmed their hybrid origin.

***Riassunto.*** Sono stati caratterizzati con un'analisi morfologica e biomolecolare cinque individui dell'ibrido naturale tra *Aceras anthropophorum* e *Orchis simia*, conosciuto come x*Orchiaceras bergonii* (Nanteuil) E. G. Cam. I caratteri morfologici sono risultati intermedi tra quelli delle due specie parentali; i dati ottenuti per lo spaziatore interno trascritto (ITS1) dei geni ribosomali hanno confermato l'origine ibrida di questi esemplari.

***Key words:*** *Aceras*, Hybridization, Orchidaceae, *Orchis*, x*Orchiaceras bergonii*.

## INTRODUCTION

Intergeneric and infrageneric hybridation within Orchidaceae is very frequent (STEBBINS & FERLAN, 1956; DANESCH & DANESCH, 1972; DANESCH et al., 1975; EHRENDORFER, 1980; STEINBRÜCK et al., 1986) and its widespread occurrence may play a significant role in speciation (EHRENDORFER, 1980; SUNDERMANN, 1980) and evolution within the family.

In the past, morphology, distribution and crossing experiments have been traditional methods of assessment in plants hybrid recognition (GOTTLIEB, 1972; STACE, 1975). Also karyology (e.g., GREILHUBER & EHRENDORFER, 1975; BIANCO et al., 1990) and more recently enzyme electrophoresis (STEINBRÜCK et al, 1986; ROSSI et al., 1992) have been adopted to trace hybrid parentage. Lately, combined approaches of protein and chloroplast DNA analysis (COZZOLINO & ACETO, 1995) and of ribosomal DNA and chloroplast DNA analysis (CAPUTO et al., 1997) have been successfully used to investigate some orchid hybrids. Infrageneric hybridation in *Orchis* L. is rather frequent; 48 hybrids for this genus have been reported (DAFNI, 1987).

In the past, members of the genus *Orchis* were grouped on the basis of morphological affinities. Cladograms for *Orchis* and related genera have been recently found by means of studies on nuclear DNA (PRIDGEON et al., 1997) and chloroplast DNA (COZZOLINO et al., 1998). In these studies, *Aceras anthropophorum* (L.) R. Br. ex Aiton fil. and *Orchis simia* Lam. resulted to belong to the same clade. The existence and the frequence of the natural hybrid x*Orchiaceras bergonii* (Nanteuil) E. G. Cam. was correlated with the weak reproductive barriers between these two entities.

In the present paper, a thorough morphological study, coupled with restriction analysis of PCR-amplified ITS1, was used to investigate the hybrid status of five plants of x*O. bergonii*. The study was accomplished by comparing the restriction fragment profiles of the presumed hybrids with those of the two species regarded as parental on morphological basis, *O. simia* and *A. anthropophorum*.

## HISTORICAL BACKGROUND

A first plant referred to the entity currently known as *xO. bergonii* was found near Muret (France) by TIMBAL-LAGRAVE (1861), who erroneously thought it a hybrid between *Himantoglossum hircinum* (L.) Sprengel and *O. simia*. Later, the

entity was reported by VAYREDA Y VILLA (1879) as *Aceras densiflora*. DE NANTEUIL (1887) found it in a locality near Paris, describing it as a hybrid between *A. anthropophorum* and *O. simia,* and named it x*Orchis bergoni*. The hybrid is also cited by RICHTER (1890) who dedicated it to E. Vayreda y Villa naming it *Aceras vayrae*; the orthography of this name was corrected by ROUY (1891) as *Aceras vayredae*. CAMUS (1892) ascribed the entity x*Orchiaceras* to a hybrid between *Aceras* R. Br. ex Aiton fil. and *Orchis*; therefore, he changed the name of the entity described by De Nanteuil in x*Orchiaceras bergoni*.

Later, this hybrid was reported by other authors, some of which described the overall morphology (ASCHERSON & GRAEBNER, 1907; NELSON, 1968; PEITZ, 1970; DEL PRETE & CONTE, 1980), others the morphology of internal tissues (CAMUS & CAMUS, 1928). The varieties described by some authors (LENDNER, 1925; CAMUS & CAMUS, 1928; KELLER et al., 1940) must be ascribed to the natural variability of the hybrid.

The original mistake of TIMBAL-LAGRAVE (1861) was repeated by CAMUS (1892) who, besides mentioning *xO. bergoni*, cited x*Loroglorchis lacazei* E. G. Camus. However this name, as well as x*Orchimantoglossum lacazeï* Aschers. & Graebn. cited by ASCHERSON & GRAEBNER (1907), was synonymyzed as x*O. bergonii* some years later (KELLER et al., 1940); now, this name is in conformity with modern rules of nomenclature (GREUTER et al., 1994).

Recently, x*Orchiaceras bergonii* and its parental species have been investigated using both morphometric analysis (BATEMAN & FARRINGTON, 1987) and morphological and molecular techniques (protein profiles and cpDNA RFLP) (COZZOLINO & ACETO, 1995).

Till now the hybrid has been reported for Great Britain, Spain, France, Switzerland, Italy and Northern Africa. In Italy, it is reported for Val Merdanzo near Bordighera (BICKNELL, 1896), Pietra alla Croce near Ancona, Giuncano between Spoleto and Terni (KELLER et al., 1940), S. Maria del Giudice near Pisa (DEL PRETE & CONTE, 1980), Lazio (ROSSI & BASSANI, 1985), and mountains W of Vallo di Diano near Salerno (COZZOLINO & ACETO, 1995).

## EXPERIMENTAL MATERIAL

Five specimens of x*O. bergonii* were examined. They were found in the course of several floristic investigations on the mountains W and E of Vallo di Diano (province of Salerno, Italy). The plants were found in a mixed population of *A. anthropophorum* and *O. simia*. Several other *Orchis* taxa [*O. coriophora* L. subsp. *fragrans* (Pollini) Sudre, *O. italica* Poiret, *O. laxiflora* Lam., *O. mascula* L. subsp. *mascula*, *O. morio* L. subsp. *morio*, *O. papilionacea* L. subsp. *papilionacea*, *O. pauciflora* Ten., *O. provincialis* Balbis, *O. purpurea* Hudson, *O. quadripunctata* Cyr. ex Ten., *O. tridentata* Scop., *O. ustulata* L.] grew sympatrically with the hybrid specimens [NAZZARO et al., 1995; 1991-1992 (1996)]. Only one voucher specimen for the hybrid entity has been deposited at NAP; for the other hybrid specimens photos are available. Voucher specimens are available at NAP for the other taxa.

Total DNA was extracted from fresh leaves using the protocol of CAPUTO et al. (1991), modified by authors.

The ITS1 region was amplified by polymerase chain reaction (PCR) using 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'). All PCR reactions (100 µl final volume) were conducted in a thermal cycle (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; 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 then purified by clorophorm-isoamilic (24:1) and ethanol precipitation. Purified fragments of hybrids and parental species were then digested with different restriction endonucleases 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.

## RESULTS

The morphological characters shown by the specimens are here reported.

Plants 13-36 cm high. Stem with 3-6 basal leaves forming a rosette and 2-3 sheaths above, naked in the upper part. Leaves lanceolate, obtuse and mucronate. Spike cylindrical, dense to rather lax, 4.2-13 cm long, 18-45 flowered, with acropetal anthesis. Bracts membranous, white-greenish, lanceolate, acute or acuminate, ½ to as long as ovary. Perianth segments forming a galea: the outer ones white-greenish or white-pinkish with purple spots and veins, ovate-lanceolate, with apexes divergent to convergent; the inner ones linear, slightly shorter than outer. Labellum 3-lobed, purple with a more or less large, white-greenish or white-pinkish basal area sometimes with purplish spots. Lateral lobes ¾ to as long as the middle one. Middle lobe larger than lateral ones, divided into 2 linear lobules, sometimes with a very short tooth in between. Lateral lobes and lobules ribbon-like, sometimes curved upwards at apex. Spur 1-2 mm, 1/5-1/7 than ovary.

The molecular analysis showed that the ITS1 PCR fragments obtained from *O. simia* and *A. anthrophophorum* were approx. 350 bp in length. As length of amplified fragments was identical in the potential parents, a restriction analysis was carried out, and only parental restriction profiles which differed from each other and which were not shared by any other sympatrically growing *Orchis* species were taken into account (data not shown).

Parental ITS1 fragments digested with *Hha* I showed a single restriction site in *O. simia*, with fragments 230 bp and 120 bp long, and two restriction site in *A. anthropophorum* with fragments 160bp, 120bp and 70bp long (the 160bp and 70bp fragments coming out from 230bp fragment). The *Hha* I digested ITS1 fragments of all the five hybrids showed an additive pattern composed of four bands (230bp, 160bp, 120bp and 70bp long) representative of all parental patterns (Fig. 1c).

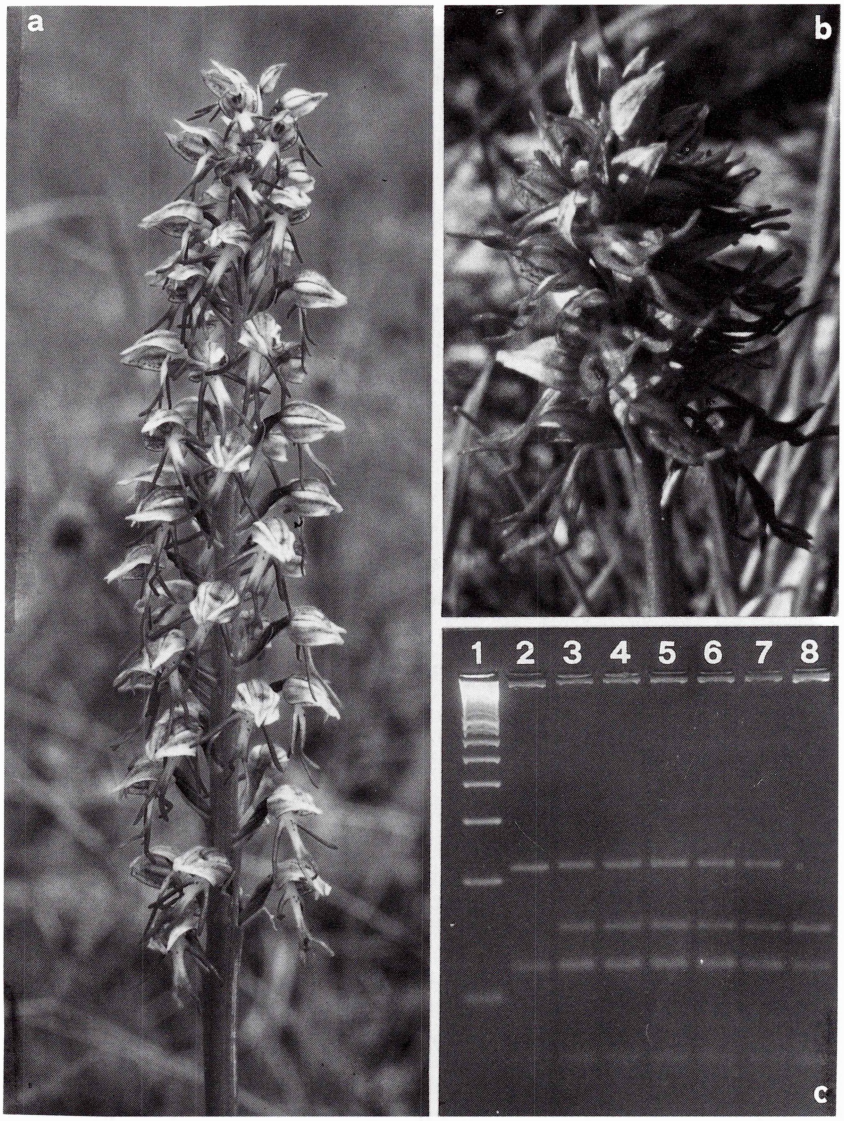


Fig. 1 - **a**, **b**) Two specimens of x*Orchiaceras bergonii* photographed in the field. **c**) Gel electrophoresis of ITS1 *Hha* I digestion of *Orchis simia* (lane 2), x*Orchiaceras bergonii* (lane 3-7), *Aceras anthropophorum* (lane 8), molecular weight ladder of 100 bp (lane 1).

## DISCUSSION

Morphological data of the five specimens found around Vallo di Diano well correspond to the diagnosis (DE NANTEUIL, 1887) and to the previous descriptions (ASCHERSON & GRAEBNER, 1907; NELSON, 1968; PEITZ, 1970; DEL PRETE & CONTE, 1980). The differences among the descriptions of these authors, often carried out on single specimens, must be ascribed to the variability of hybrids. In the present description, all the variation reported by previous authors occurs. The only absence is the purplish galea described by DEL PRETE & CONTE (1980).

The specimens generally show intermediate morphology between the parental species. Morphological characters are sometimes closer to those of one parent; for example, Fig. 1a shows a specimen with *Aceras*-like inflorescence, while fig. 1b shows a *O. simia*-like inflorescence.

In all specimens, the typical characters of the parental species are the acropetal anthesis, as in *A. anthropophorum*, and the presence of the spur, as in *O. simia*.

For a careful comparison between the morphological traits of the hybrids and those of the parental species, see Tab. 1.

ITS restriction patterns of the hybrids, in which all parental bands are present, clearly indicate that *O. simia* and *A. anthrophoporum* provide parental lineages (as inferred by morphology). The *Hha* I sites in the parental species have been selected among other restriction enzyme sites investigated because their patterns are exclusive of the aforementioned species. All restriction sites are present also in all the examined hybrid progeny. Further, the hybrid pattern shows visually similar amplified quantities of the ribosomal DNAs of the two parents. The absence of any predominant parental DNA patterns leads us to the conclusion that all the hybrids may represent F1 progenies or, at most, the result of a cross between two hybrids. In fact if a back-cross occurs, the ratio between parental DNA in the hybrids is shifted in favor of one parental species.

Tab. 1 - Synopsis of the main morphological characters of *xOrchiaceras bergonii* individuals found around Vallo di Diano and their putative parents.

| | **Anthesis** | **Spike** | **Bracts** | **Outer perianth-segments** | | **Color of labellum** | **Spur** |
|---|---|---|---|---|---|---|---|
| | | | | **Apex** | **Color** | | |
| *Orchis simia* | Basipetal | Ovoid or broadly cylindrical, dense | Usually ½ as long as ovary | More or less divergent | White with purple spots and veins | White with purple spots and lobe apex | More or less 1/2 as long as ovary |
| *xOrchiaceras bergonii* | Acropetal | Cylindrical, dense to rather lax | ½ to slightly shorter than ovary | Divergent to convergent | White-greenish with purple veins | White-greenish with purple spots and lobe apex | 1/5 – 1/7 as long as ovary |
| *Aceras anthropophorum* | Acropetal | Narrowly cylindrical, dense to rather lax | Shorter or slightly shorter than ovary | Convergent | Yellow-greenish with reddish streaks | Yellow-greenish, sometimes with reddish-brown margins | Absent |

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