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Identification of Two Fungal Endophytes Associated with the Endangered Orchid *Orchis militaris* L.

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A survey of the endangered orchid *Orchis militaris* populations was carried out in north-eastern Italy. The occurrence of fungal root endophytes was investigated by light and electron microscopies and molecular techniques. Two main sites of presence were individuated in the Euganean Hills, differing as to the percentage of flowering individuals and of capsules completing maturity. Fluorescence microscopy revealed an intracellular cortical colonization by hyphal pelotons. Two ITS PCR products co-amplified. Sequencing revealed for the former an identity and a high similarity (99%) with a Tulasnellaceae (Basidiomycota) fungus found within tissues of the same host in independent studies in Hungary and Estonia, suggesting an interesting case of tight specificity throughout the Eurosiberian home range. The second amplicon had 99% similarity with *Tetracladium* species (Ascomycota) recently demonstrated as potential endophytes. TEM revealed two different hyphal structures. Double fungal colonization appears to occur in *Orchis militaris* and the possible requirement of a specific fungal partner throws light on the causes of this plant's rarity and threatened status.

Keywords: *Orchis militaris*, endangered taxa, Euganean hills, mycorrhizal symbionts

The majority of vascular plant species engage in mutualistic interactions of trophic nature with defined taxa of fungi. The resulting association is broadly referred to under the collective term of mycorrhiza [7]. Recently reviewed [3], this definition encompasses a number of morphofunctional types involving different plant families and corresponding fungal groups with variable degrees of specificity. Among these types, the orchid mycorrhiza is a category with important peculiarities, as the fungi often sustain the life of these small-seeded plants through the delicate offspring stage and begin receiving carbon only once the plant is established [17, 28]. Some orchid species however remain achlorophyllous throughout their adult phase and persistently exploit their mycorrhizal fungus without a mutualistic return of organic carbon [29]. The biology of orchid mycorrhiza has been thoroughly reviewed [5, 21]. Different methods have been used to investigate these interactions; microscopy has been for a long time the primary approach [9]. The use of fluorescent stains has helped visualizing root invasion in optical microscopy [20, 22]. The advent of biomolecular techniques has allowed to verify the fungal identity with high degree of accuracy [8, 13, 15]. These studies, based on sequence comparison of the ribosomal operon region, have pointed out that the traditional association of fungi of the Rhizoctonia group (phylum Basidiomycota; subphylum Agaricomycotina) as the typical orchid symbionts [28] has several exceptions. Selosse *et al.* [24] showed that both achlorophyllous and photosynthetically active specimens of *Epipactis* are in mycorrhizal interaction with truffles (i.e., ascomycetes of the genus *Tuber*). Girlanda *et al.* [10] found that the Mediterranean orchid *Limodorum abortivum*, another member of the Neottiae tribe, was predominantly associated with basidiomycetes belonging to the *Russula* genus. In the present investigation, we focused our attention on the soldier's orchid *Orchis militaris*, an endangered Eurosiberian species whose occurrence into the Mediterranean regions is rare and localized [6]. The plant is also recorded as sporadic in Britain and confined to a few sites in Sussex, Kent, and Buckinghamshire [4]. Among the reasons that might have led to its threatened status, the presence and diffusion of specific fungal symbionts can be one of the possible limiting factors. In the northern side of its host range, this plant's fungal associations have been investigated in Estonian mine soils [27]. As pointed out by a number of evidences, the issue that mostly determines the distribution range and the persistence of plant species, once climatic requirements are met, is the below-ground interaction with

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specific microorganisms [14, 30]. Accordingly, plants ending up in red lists may be suffering a limitation imposed by their microbiota population dynamics, whose status is in turn affected by environmental pollution, soil management practices, and land usage in general. In this study, we aimed at verifying whether *O. militaris* was endowed with a mycorrhizal colonization in the natural habitats under study, and, if that were the case, which was the identity of its associated fungi. In the first instance, we performed a botanical survey on this rare plant distribution in an area located in north-eastern Italy, which we coupled to the analysis of its root associations, using both microscopy- and DNA-based approaches.

MATERIAL AND METHODS

Plant Distribution in the Study Area

The occurrence of *O. militaris* in the area of Euganean Hills was surveyed and a census of its populations and their productivity was made by recording the following: number of specimens, number of fruits per specimen, and number of pods reaching maturity per specimen. Descriptive statistics was computed (mean, median, standard deviation) and differences among sites were evaluated by the Kruskal–Wallis one-way analysis of variance by ranks test.

Plant Collection, Root Tissue Processing, and Epifluorescence Microscopy

Orchis militaris whole specimens were collected at the flowering stage in mid-May on Mt. Lozzo, in the Euganean Hills, north-eastern Italy (45°17'47"N; 11°37'12"E). Plants were excavated with a clod of their surrounding soil and transferred to the laboratory. Root apparati were carefully cleaned from the soil under running water. Free-hand sections of both roots and tubers were obtained. Cylindrical portions were transferred into 1.5-ml polypropylene conical tubes to be used for DNA extraction and stored at −20°C. Free-hand cross-sections for light and fluorescence microscopies were stained in acridine orange 0.05% [26] for 10 min, rinsed with distilled water, and placed on microscopy slides. These were visualized under an Olympus BX60, equipped for epifluorescence with a mercury lamp. Digital images were acquired by an Olympus Camedia C3040 camera.

Light and Electron Microscopies

For light and electron microscopies, small pieces of roots were excised and fixed overnight in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 6.9). Samples were post fixed in 1% osmium tetroxide, dehydrated in a gradient series of ethanol and propylene oxide, and embedded in Epon-Araldite. Semi-thin sections (1 μm) were stained with 1% Toluidine Blue. Ultrathin sections were observed, after uranyl acetate and lead citrate staining, with a Hitachi H 300 EM operating at 75 kV.

DNA Extraction and PCR Amplification of Ribosomal Intergenic Spacer

Samples obtained as described above were thawed, and 50 μl of sterile TE buffer (10 mM Tris·HCl, 1 mM EDTA, pH 8) was added to the tubes, and root portions were mashed by means of flame-sterilized forceps tips. The tubes were subsequently incubated for 30 s in a microwave oven set at 700 W. The procedure was repeated twice. Samples were allowed to cool at room temperature for 5 min and centrifuged for 10 s to pellet plant debris. One μl of supernatant was withdrawn and used as template for the PCR reaction. Primers used included ITS1, ITS1F, and ITS4 [32]. The following primer pair combinations were tested: ITS1–ITS4 and ITS1F–ITS4.

One μl of the lysate containing the total DNA was treated in a PCR BioRad I-Cycler using primers at 1 μM each in a 25-μl reaction volume, and adopting the following program: initial denaturation at 95°C for 2 min; 30 cycles at 94°C for 1 min, 45°C for 1 min, 72°C for 1 min; and a final extension at 72°C for 7 min. The PCR reaction mixture contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dATP, dCTP, dGTP, and dTTP, 1 μM of each primer, and 2.5 U *Taq* DNA Polymerase, recombinant (Invitrogen Life Technologies). Amplification products were visualized by loading 5 μl from the PCR reaction on a 1.5% agarose gel in 0.5× TBE buffer [18], run electrophoretically for 3 h at 110 V. The ethidium bromide-stained gel was visualized over an UV transilluminator and photographed by a Kodak DC290 digital camera. In cases where multiple bands were observed, each was separated by cutting the gel slice containing each band over the transilluminator and purifying the DNA by means of a QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany). The purified material was used as template for a further round of PCR amplification using the same condition, and band purity was verified by electrophoresis as described above. For an alternative to gel slicing and Qiaquick purification, the DNA band in the gel was simply touched by penetrating the agarose with a sterile plastic pipet tip to be subsequently dipped into the PCR reaction mix.

DNA Sequencing

One μl of the solution resulting from the above-described PCR amplification was mixed with 1 μl containing 6.4 picomoles of the forward primer, used in 0.2-ml polypropylene tubes, and then dried by incubating the open tubes for 15 min at 65°C in an I-Cycler thermal cycler. A parallel reaction was performed separately using the reverse primer. The template and primer mix was directly used for dideoxy-cycle DNA sequencing with fluorescent terminators (Big Dye, Perkin-Elmer/Applied Biosystems, Foster City, CA, U.S.A.) and run in an Applied Biosystems ABI Prism 3730XL automated DNA sequencer. Chromatograms were analyzed by Chromas 2.23 software (Technelysium Pty Ltd, Tewantin, Australia). Merging of the complementary strands was achieved by Lasergene software v.7.2 (DNASTAR Inc., Madison, WI, U.S.A.) and the similarities to database records were investigated online through the NCBI platform (http://www.ncbi.nlm.nih.gov) using the BLAST utility. A neighbor-joining tree against selected database sequences was constructed using the software Mega v. 4 [16].

RESULTS

Plant Occurrence and Population Data

From the two sites investigated, 44 individuals were found on Mt. Cero and 101 on Mt. Lozzo. Of these, 48% and 65% had flowers, respectively, and 32% and 56 % bore fruits. The latter site appeared to offer more favorable conditions,

sustaining a larger population that performed better in terms of both flowering and fructification. Descriptive statistical analyses further underlined the higher productivity of the Mt. Lozzo site; the right tail of the bell-shaped distribution of the flower number contained a conspicuous number of individuals bearing a high number of flowers per plant (32–34), whereas on Mt. Cero the maximum value of flowers recorded per plant was 24. The minimum numbers observed instead were 14 and 2 flowers per plant, respectively. The Kruskal–Wallis test indicated a statistically significant difference between the two stations in terms of number of flowers per specimen but not for the number of pods reaching maturity. As regards habitat, *O. militaris* appeared to prefer the ecotonal border between thermophilic oak woods and arid meadows.

Microscopic Approaches Reveal Different Fungal Structures Colonizing *O. militaris* Roots

Six plant specimens, three from each of the two sites, were dissected and analyzed. Microscopy clearly showed evidences of internal root colonization in samples from both sites. The brightfield image (Fig. 1a) showed how a large crown of the cross-section (mostly visible on the right side) was cluttered by cell-filling pelotons within the cortex. Blue light excitation of acridine orange incubated sections revealed that the filling material stained accordingly, further supporting its fungal nature. Fungal mycelium appeared confined to the cortex and only marginally approaching the central stele in which the yellow-staining vascular bundle was visible (Fig. 1b). Details of mycelial glomerular structures coiling inside cells are visible in Fig. 1c. As regards other portions of the root apparatus, as the storage tubers were stained with the same procedure and proved devoid of mycelia, cells were filled only with nonstaining amyloplasts (data not shown). Light microscopy on thin sections, obtained from tissues close to the root portions used for molecular typing, confirmed an intracellular colonization with fungal hyphae. In the outer cortical cells under the root epidermis, hyphae occupied all the cell and were unclumped, freely running in all directions (Fig. 2a, 2e, and 2b). In the inner cortical cells, glomerular structures made of thin clumped hyphae were usually visible in the center of the cell (Fig. 2a and 2c). Transverse sections allowed to appreciate a massive occurrence of hyphae in the intercellular spaces between outer cortical cells (Fig. 2d). They appeared to be of different morphology, suggesting the occurrence of at least two fungi. This was confirmed by the electron microscopy: intercellular spaces harbored hyphae having rather different sizes, shapes, and cellular contents (Fig. 2f). In the same zone, the hyphae into the cytoplasm of the cortical cells presented septa that separated fungal cells rich in electron transparent lipid bodies (Fig. 2g, 2h, and 2i). The host cell membrane always surrounded the hyphae and a thin layer of electron-dense interfacial material was observed between the latter and the host cell membrane (Fig. 2g, 2h, and 2i, arrows). The septa in these hyphae are of basidiomycetous type; in Fig. 2g (longitudinal view) and Fig. 2i (front view), the presence of entire parenthesomes (arrowheads) and cell wall bulges at the edge of the pore clearly revealed the typical dolipore structure. By electron microscopy, it was not possible to define which fungal type the clumped hyphae, building pelotons in the inner cortical cells, belonged to. Pelotons, surrounded by host cell membrane, were constituted by randomly distributed very thin hyphae, and no septa types were recognizable (Fig. 2e). Some fungal hyphae appeared collapsed (Fig. 2f).

PCR Amplification of Fungal ITS Region

The same number of specimens used for microscopy was subjected to molecular analyses. Extraction of cell content from root tissues and polymerase chain reaction amplification consistently gave rise to amplicon electrophoretic profiles displaying two bands of variable reciprocal intensity running at positions corresponding to 800 and 600 bp (hereafter designated band 4b1 and 4b2, respectively). Each of the two resolved bands was purified by extraction from the gel and reamplification under the same conditions. Amplicon purity was ascertained again by electrophoresis and DNA sequencing was carried out. Results were the following: band 4b1 (800 bp) (GenBank code EU490419)

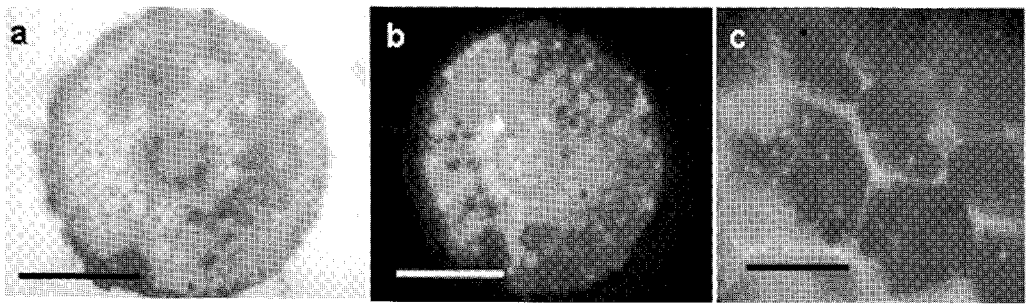

Fig. 1. Root cross-sections of *Orchis militaris* stained with acridine orange.
(**a**) Brightfield observation; (**b** and **c**) fluorescence images obtained using a WIB filter. Fungal pelotons staining orange-red are visible in over half of the cortex cells. Details on the glomerular structure of the hyphal coils are shown in *c*. Scale bars: 500 μm (**a**, **b**), 50 μm (**c**).

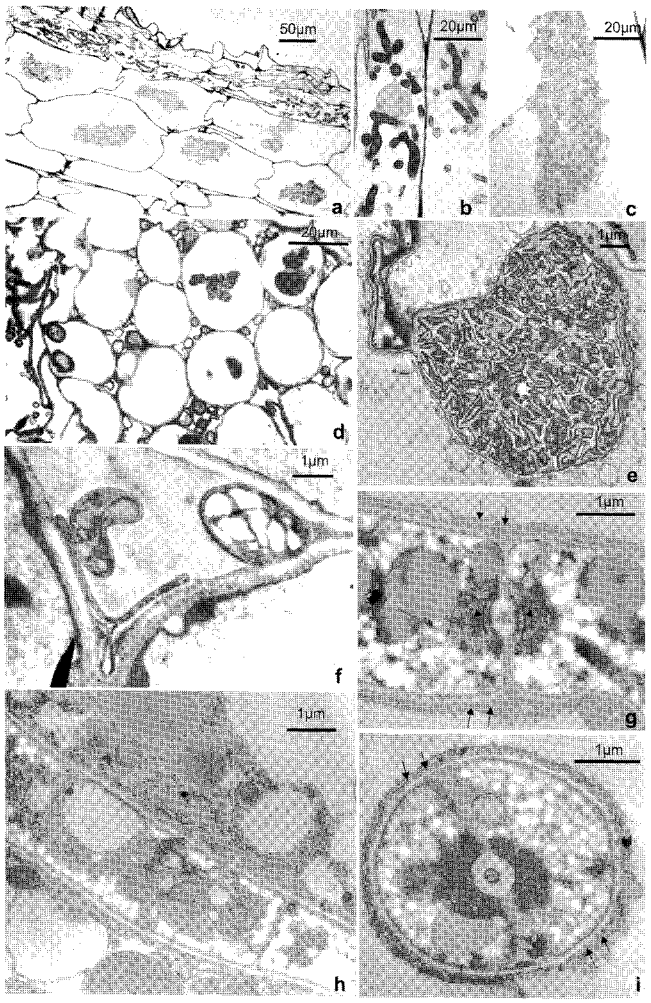

Fig. 2. Light and electron microscopies on sections of *O. militaris* roots, showing fungal colonization.
(**a**) Longitudinal section showing hyphae in the inner and outer cortical cells. (**b**) Outer cortical cells filled with unclumped hyphae; (**c**) inner cortical cell showing glomerular structures made by thin clumped hyphae; (**d**) cross-sections of a root showing hyphae in the intercellular spaces between outer cortical cells. (**e–i**) Transmission electron micrographs showing a fungal peloton surrounded by the host cell membrane (**e**); hyphae of different size and shape in an intercellular space (**f**); in the outer cortical cells, hyphae are rich in lipid bodies into the cytoplasm (**h**). Longitudinal (**g**) and frontal (**i**) sections of a dolipore septum with entire parenthesomes (**g**, arrowheads) and cell wall bulges; arrows in *g* and in *i* indicate the host cell membrane around fungal hyphae.

shared 100% similarity with sequences from a series of uncultured fungi from the roots of the same species of *O. militaris* in Hungary (AM711604–AM711613, unpublished), and 99% similarity with an uncultured fungus ascribed to the family Tulasnellaceae (Basidiomycota) found again in roots of *O. militaris* in Estonia (EU195344) [27]. Aside from this set of very related database entries, the further nearest similarities were separated by a substantial gap, scoring not more than 84% with isolates of *Epulorhiza* sp., values in the range between 81% and 86% with uncultured Tulasnellaceae, and 81% with a sequence (AJ549121) from

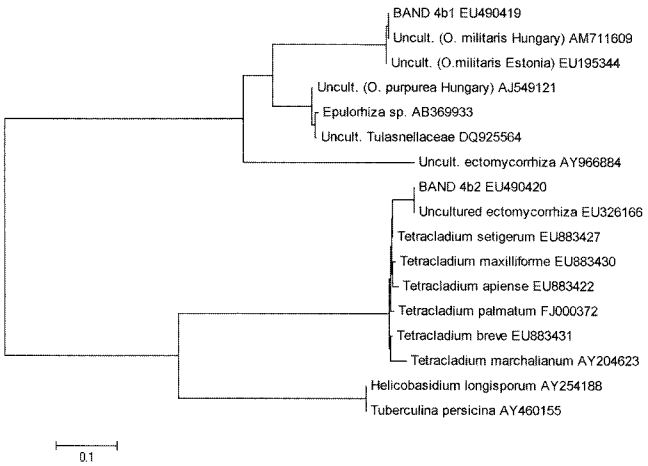

Fig. 3. Neighbor-joining clustering alignment tree showing the phylogenetic relationships between the two amplicon sequences obtained from *O. militaris* roots (bands 4b1 and 4b2) and selected database relatives.
GenBank accession codes are indicated. The optimal tree with the sum of branch length=2.01056074 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option).

an uncultured fungal symbiont of *Orchis purpurea*. Lower colinearities were shared with ectomycorrhizal basidiomycetes (AY966884), *Helicobasidium longisporum* (AY254188), and *Tuberculina persicina* (AY460155).

As regards band 4b2 (600 bp, GenBank code EU490420), we recorded a 99% identity with an uncultured ectomycorrhizal *Tetracladium* (mitosporic Ascomycota) found within *Salix herbacea* roots on a glacier of the Austrian alps (EU326166), 98% identity with an uncultured ectomycorrhizal ascomycete found in truffle grounds (AJ879646), and values in the 96–97% range with a series of different *Tetracladium* species including *Tetracladium maxilliforme* from mycorrhizal root tips of *Pinus sylvestris* (DQ068996). An identity of 96% was recorded with an uncultured ectomycorrhizal ascomycete isolate from the orchid *Cephalanthera damasonium* (AY833029). A neighbor-joining cladogram including some of the nearest database relatives of the two sequences was elaborated and is shown in Fig. 3.

DISCUSSION

Two different fungal taxa, both with affinities to known plant associative fungi, have been found within the roots of *Orchis militaris*. The first belongs to the phylum Basidiomycota, and the second to the Ascomycota. Only the former is therefore within the rank that includes the *Rhizoctonia sensu lato* group, traditionally indicated as the fungal symbionts of orchids. For this fungus, indicated

by DNA homology as a *bona fide* member of the Tulasnellaceae family, a marked instance of host specificity arises from database matches. Indeed, the only cases of significant homology (99%, which is within the species boundary) pertain to isolates from roots of *Orchis militaris* collected in two geographical locations (Estonia and Hungary) very distant from the one of the present work. It is interesting to note the extent of fungal species consistency across the boundaries of this Eurosiberian plant range, as well as the restriction of this fungus association to *O. militaris*, as far as present databases allow to assess. This observation indicates how a possible constraint of a specific mycorrhizal partner could be operative for *O. militaris*. According to the literature [5], specificity for fungi by orchids can lead to higher rates of seed germination and a more efficient physiological association. Orchids undergoing prolonged dormancy periods, or those that are confined to shady habitats, are postulated to have a higher dependency on fungal carbon than evergreen or annually flowering plants, or those growing in exposed habitats [10]. As a consequence, the former can have an advantage from an efficient specific association. Fungal specificity and orchid rarity may also be correlated when the fungal partner is itself rare or distributed patchily in the environment. The consistency of identity at the species level (99%) with isolates from the same plant collected in Italy, Hungary, and Estonia suggests that this species, which is particularly threatened and rare in the southern part of its home range, could have a particularly tight host-specificity constraint. Many examples in the literature indicate that orchids and fungi associate through compatibility barriers whose nature is not entirely known. A narrow level of specificity in an orchid is envisaged as a possible reason for its rarity and vulnerability, just as a narrow food preference would in an animal species [21].

The second fungal species that co-amplified is reminiscent of the findings commented by Selosse *et al.* [23] on the unexpected but arising occurrence, as plant endophytes, of the so-called Ingoldian fungi (aquatic hyphomycetes whose teleomorphs are classified within the Ascomycota). *Tetracladium* is one of the most recurring cases for these species that appear to spend part of their cycle as water fungi, but have the capability of entering plants, including orchids. It has been postulated that these asexual aquatic hyphomycetes could exploit two niches in their lifestyle, moving from the water into plants, wherein endophytism could possibly allow the onset of their sexual stages [23]. *Tetracladium* sequences have been found also in roots of the orchid *Cephalanthera longifolia* [1]. *Tetracladium*-related sequences were also found in a survey of ectomycorrhizal fungal communities in stands of *Tuber magnatum* [19].

Among the novel aspects of the present work stands the fact that two fungi have been found to be associated with the same orchid host plant. Such dual infection of the same roots used to be considered an infrequent finding in orchid–fungi interactions [15]. The two phylogenetically distant taxa appear to share closely spaced tissues, as their amplicon bands arose within the same PCR reaction. The intensity of their amplification bands was about equal, indicating similar biomass and template abundance with no strong dominance of one of the two within the plant tissues. The presence of two fungi in association with the same plant does not necessarily prove that both play a trophic role of mycorrhizal nature. One likely interpretation is that the first could be the custom mycorrhizal partner, whereas the second could be an endophyte whose possible beneficial (or merely commensal) role remains to be established. Occurrence of endophytic microfungi in plant roots and stems, including orchids, is reported [3], but an unsolved issue standing out in these kind of studies is indeed the difficult distinction between orchid mychorrhizal and orchid endophytic fungi [2]; the former being ascertained mutualists and the latter simply microorganisms growing inside plant tissues without causing symptoms. Possibilities exist that, in case of dual presence, one of the fungi could be in mycoparasitic relation towards the other [31]. There is also a possibility that additional, yet to be detected, species could also be present and that the interactive picture could be even more complex.

Using the microscopy approach as complement to molecular-based indications, we can visually confirm the location of least one of the two kinds of fungi, as dolipore-bearing septa, characteristic of Basidiomycota were clearly evidenced. The possible co-presence of the Ascomycota in the root could nevertheless be consistent with the two different hyphal types observed in intercellular spaces, as judged by their size and cellular content. In addition, it can be underlined that the abundant central pelotons and coils cannot be tributed univocally to any of the two taxonomical divisions owing to the degenerate state of these hyphae, which is typically reported to occur in orchid mycorrhizae [12, 15, 24]. These could therefore either belong to the second taxon, indicated by the molecular analyses, or represent a differentiated stage of the first one.

In terms of plant fitness, the site on Mt. Lozzo appears to offer remarkably better conditions, yielding a more than double population. In both sites, however, the number of maturing pods was equal to about 50% of the flowers. The plant does not thrive inside woods either, where it is supposedly limited by low light, nor in the dry open fields, where it endures competition for nitrogen and water from better-adapted vegetation. It appears to strictly depend on the ecotonal transition zone. A moderate human or animal disturbance (hay reaping, pasture, recreational activities) appears to be of help, as it limits forest spread and reduces the vigor of competing meadow species. The presence of rocky outcrops and calcareous gravel from sedimentary depositions is also important, as it originates bare ground

spaces that are particularly suitable for *O. militaris* growth. The discussed results, constituting the first report on the possibility of a double myceliar interaction with roots of *Orchis militaris,* enable to cast some further light on the biology of this endangered species and to speculate on the reasons of its rarity in a considerable part of the home range. Studies in the literature have examined habitats and conditions limiting the diffusion of related orchids such as *Orchis simia* [33], a population of which, originating from a single individual, was followed in The Netherlands for several years. Juveniles would appear only four years after the mother plant had flowered and the overall spread of the species was slow, amounting to a total of 65 individuals in 10 years. Mycorrhizae are regarded as a critical issue in overall terrestrial plant survival. To some authors, they could be envisaged as the key of the plants' success in land colonization [14]. Considering the dependence of orchids on these interactions in overcoming the offspring stage, and having ascertained the presence of mycorrhizal taxa in *O. militaris*, we could hypothesize that their availability through soil could be a factor limiting this *Orchis* establishment and diffusion. The need for two different fungi, if both were critically involved in mutualistic interactions, could further reduce the environmental chances for *Orchis militaris*, contributing to explain its critical status. The requirement could also be reciprocal, in that the distribution and persistence of the fungi themselves could require the plant presence; it has long since been reported that orchid mycorrhizal fungi are hardly ever isolated from soil far from their hosts [11], although orchid seed baiting methods have in part counteracted such a view.

An additional factor that is to be considered in interpreting a plant species distribution is the possibility of being connected through a myceliar net to one or more plants. Such phenomena, defined under the term of Common Mycorrhizal Network, are earning an increasing awareness by the scientific community. An important consequence of the hyphal web is the possible transfer of nutrients among plants. In this respect, even green chlorophyllous orchids have been shown to obtain organic carbon from other plants *via* the fungus to an extent reaching values up to 85% [25]. Concerning such possibilities, it is to be reported that *O. militaris* stems were consistently more numerous when found near woody shrubs of *Cotynus coggygria* or *Viburnum lantana* on the Mt. Cero site. The second location examined, Mt. Lozzo, where *O. militaris* numbers were more than double, is also more densely covered by bushy vegetation and profuse groups of the orchid individuals were typically spotted near *C. coggygria*, *Ostria carpinifolia*, *Quercus pubescens*, or *Rubus ulmifolium*.

The observations presented suggest that, to plan actions for the conservation of this kind of endangered plant species, an integrated approach has to be recommended. The ecological analysis can benefit from the microbial ecology-based perspective in trying to individuate possible limiting resources of biotic nature. The possibility that more than one fungal endophyte is required to fully sustain plant development could provide novel insights in interpreting its environmental outcome. Future work will be devoted to assess which fungal symbionts do associate with other orchids of different abundance and status in the same area. In addition, attempts to isolate and cultivate the fungal endophytes will be carried out to provide the possibility of *in-situ* land inoculation with the mycorrhizal symbionts. This practice will be tested in the pursuit of enhancing the survival and fitness of these endangered orchid species.

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