Mycorrhizal fungi and endophytes of orchids living in three Hungarian abandoned mines

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Several ascomycetous and basidiomycetous fungal genera have been identified from the roots of orchids, with which they might have a mycorrhizal relationship. This study investigates the fungal partners of orchids living in Hungarian abandoned mines. Individuals of one to three orchid species were collected in three Hungarian abandoned mines (Pusztavám, Tokodaltáró, Székesfehérvár). DNA was extracted from isolated fungal strains or directly from surface-sterilised orchid roots. The nuclear ribosomal internal transcribed spacer (ITS) region of fungal DNA was amplified in a polymerase chain reaction (PCR). Sequenced ITS regions were identified based on the most similar sequences in the GenBank nucleotide database (National Center for Biotechnology Information, NCBI). Besides fungal genera considered to be orchid symbionts (*Epulorhiza* spp., *Ceratobasidium* spp. and *Sebacina* spp.), sequences similar to *Coprinopsis atramentaria*, *Fusarium* strains, dark septate endophytes and a member of Pezizales were found. This is the first time that *C. atramentaria* has been found in the roots of photosynthesising orchids. The fungal clade *Epulorhiza 2* might contain disturbance-tolerant fungi. The fungal specificity of *Orchis militaris* seems to be lower than previously assumed.

Keywords: disturbed habitat, *Epulorhiza*, orchid mycorrhiza, specificity.

The Orchidaceae are one of the most diverse plant families, comprising more than 25000 species all over the world (Dressler 1993). Members of this family associate with appropriate fungal partners to form a special type of mycorrhiza, known as orchid mycorrhiza, as the dust-like seeds of orchids contain scant energy reserves. During germination the seeds are mycoheterotrophic, i.e. their fungal partners provide the essential nutrients for them. This obligate dependence becomes facultative as the orchids begin to photosynthesise, although some orchid species with reduced photosynthetic ability remain dependent upon their mycorrhizal partners to a greater or lesser extent (e.g. mycoheterotrophic and mixotrophic orchid species) (Smith & Read 2008).

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Based on earlier studies, many orchid mycorrhizal fungi are considered to belong to the form genus *Rhizoctonia*, a basidiomycetous group of fungi (Roberts 1999). Since the isolates of orchid mycorrhizal fungi are usually sterile in culture and it is very hard to induce teleomorphs, the identification of anamorphic genera (*Epulorhiza*, *Ceratorhiza* and *Moniliopsis*) was based on the morphological characteristics of the mycelia, hyphae and monilioid cells (Currah & Zelmer 1992). Moore's (1987) system links the identified anamorphic genera with their teleomorphic equivalents as follows: *Epulorhiza* with *Tulasnella* or *Sebacina*, *Ceratorhiza* with *Ceratobasidium* and *Moniliopsis* (including *Rhizoctonia* s. str.) with *Thanatephorus* or *Waitea*.

In recent years many other basidiomycetous and ascomycetous fungal genera have been identified from orchid roots due to the use of molecular methods (Selosse *et al.* 2004, Yamato *et al.* 2005, Stark *et al.* 2009), although the function of these fungi (i.e. mycorrhizal, endophyte or pathogenic) is uncertain in many cases. In vitro germination trials and microscopy are the most convenient tools to determine whether a fungus is mycorrhizal or not. It has become clear that mycoheterotrophic orchids associate with ectomycorrhizal fungi, such as members of the Russulaceae, Coprinaceae or Thelephoraceae (Taylor & Bruns 1999; McKendrick *et al.* 2000, 2002; Yamato *et al.* 2005), to supply their nutritional requirements. Also many putatively photosynthetic orchids have been proven to live in symbiosis with ectomycorrhizal fungal species, gaining nutrients from the surrounding trees in a tripartite symbiosis (Bidartondo *et al.* 2004).

Few studies have examined the orchid mycorhizal fungal communities of various habitat types (Kottke *et al.* 2007, Illyés *et al.* 2009), especially those of mines. It was previously established that orchids may be abundant in disturbed habitats, e.g. in abandoned mines, abandoned vineyards and along paths (Molnár 2011). Shefferson *et al.* (2008) studied the mycobionts of three orchid species living in Estonian mine tailings hills, and found that the mycorrhizal interactions did not vary with the habitat type. The investigated orchid species had specific mycorrhizal partners from the fungal taxa Tulasnellaceae (*Epipactis atrorubens* and *Orchis militaris*), Ceratobasidiaceae (*Dactylorhiza baltica*) and Pezizales (*E. atrorubens*). An Australian researcher team (Bonnardeaux *et al.* 2007) also investigated the diversity of the mycorrhizal fungi of terrestrial orchids in diverse habitats, including mine rehabilitation sites. The fungi identified from *Disa bracteata* in mine rehabilitation sites were *Tulasnella* sp., *Epulorhiza* sp. and an ericoid mycorrhizal species.

Mycorrhizal specificity was a controversial topic for many years (Hadley 1970, Warcup 1971). As a solution to this problem, Masuhara & Katsuya (1994) suggested the use of the terms 'potential specificity' and 'ecological specificity', which makes a distinction between *in vitro* and *in situ* specificity, the former being wider. It is generally considered that mycoheterotrophic orchids have more specific relationships than photosynthetic ones (Taylor & Bruns 1999, McKendrick *et al.* 2002, McCormick *et al.* 2006), although there

are exceptions, where photosynthetic orchids associate with a narrower or similar range of fungi than do mycoheterotrophic orchids (McCormick *et al.* 2004, Otero *et al.* 2004).

Recent studies indicate that the level of specialization depends on the environmental circumstances. Many studies found that different mycorrhizal fungi colonized the roots of the same orchid species at various habitats (Perkins *et al.* 1995, Ogura-Tsujita & Yukawa 2008, Illyés *et al.* 2009). It has been shown that fungal diversity, which is a crucial factor for the germination of orchid seeds, is influenced by plant-fungal interactions (Perkins & McGee 1995, Kottke *et al.* 2007), resource availability (Waldrop *et al.* 2006) and the water supply of the habitat (Illyés *et al.* 2009).

Evidence is accumulating that mycorrhizal fungi are not equivalent functionally (McCormick *et al.* 2004; Otero *et al.* 2004, 2005). Orchids are considered to choose between two strategies: (1) specialist orchids associating with a narrow range of fungi have more effective germination and resource supplies, but are highly dependent on the presence of the appropriate fungus, which could easily result in patchy distribution or reproductive isolation (2) generalist orchids associating with a broad range of fungi have less effective germination and resource supplies, but are more viable under changing conditions (Dearnaley *et al.* 2007). It is suggested that the generalist strategy is favourable in varying environmental circumstances (Otero *et al.* 2004). However it is also stated that in the case of environmental stress orchid mycorrhizal interactions become specialized (McCormick *et al.* 2006).

The present study aimed at identifying the mycobionts of orchids living in three Hungarian abandoned mines, with special regard to the species-specificity of *Orchis militaris*. A further question was whether the orchid mycobiont communities were different from those observed in natural habitats (earlier investigations, see Illyés *et al.* 2009) and whether there were any differences in the composition of orchid mycobionts in the three mines.

Materials and methods

Orchid species and study sites

Field examinations were performed in three Hungarian abandoned mines. Orchid sampling efforts were limited because all the Hungarian orchid species are protected, so only 6–7 orchid individuals were sampled at each mine. (1) The mine near Pusztavám is an abandoned coal-mine, where *Orchis militaris* is the only orchid species present. The habitat is an open forest resulting from secondary succession, *Pinus silvestris* being the most abundant tree species, with *Sanguisorba minor* and *Hieracium pilosella* dominating in the undergrowth. Seven plants of *O. militaris* were collected during the flowering period of the orchids, in the spring of 2006, 2008 and 2009. (2) The mine-site near Székesfehérvár is an abandoned sand-pit. Secondary succession is in a more advanced stage than in Pusztavám, so nine orchid species were found here (*O. militaris*, *Orchis coriophora*, *Orchis laxi-*

flora ssp. *palustris*, *Orchis morio*, *Cephalanthera damasonium*, *Cephalanthera longifolia*, *Dactylorhiza incarnata*, *Epipactis palustris* and *Ophrys sphegodes*). Three sample sites were chosen in the sand-pit. At sample site 1, which is a pioneer open sand habitat with *Molinia* mosaics, *Bothriochloa ischaemum* and *Schoenus nigricans* is the most abundant plant species, two flowering plants of *Orchis coriophora* and two of *O. laxiflora* ssp. *palustris* were sampled during the years 2008 and 2009. At sample sites 2 and 3 which are *Molinia* meadows, *Molinia caerulea* and *Schoenus nigricans* are the most characteristic plant species. At sample site 2 a *Dactylorhiza incarnata* plant was collected in 2008, while at sample site 3 one *Orchis militaris* plant was studied in 2009. (3) The mine located near Tokodaltáró is also an abandoned sand-pit, which provides habitats with different water regimes. The mine-site is inhabited by four orchid species: *O. militaris*, *Dactylorhiza incarnata*, *Epipactis palustris* and *Listera ovata*. This mine also has more diverse vegetation than Pusztavám. Two sample sites were chosen, one is a dry habitat, where one *O. militaris* plant was sampled in 2006, and where *Calamagrostis epigeios* and *Ononis spinosa* are the most abundant plant species. The other sample site is a wet *Betula pendula* forest with *Equisetum* × *morei* in the undergrowth. Two plants of *O. militaris*, one of *E. palustris* and two of *D. incarnata* were collected here in the spring of 2006 and 2009.

Identification of fungal endophytes and phylogenetic analysis

The roots of the collected orchids were used for the identification of fungal endophytes. In 2009, fungal strains were isolated from orchid roots. The orchid roots were washed with tap water and surface sterilised by immersion in 0.1 % $AgNO_3$ solution for 3 minutes, after which 1 cm long root sections, cut longitudinally were placed on PDA medium (Potato Dextrose Agar, 3 g m^{-3} potato flakes, 10 g dm^{-3} glucose, 15 g dm^{-3} agar) with the cut surface down. Depending on the extent of the root system, 3–9 roots were examined per plant. Roots were handled separately, in order to avoid repeated sampling of the same fungal colony. Growing fungal colonies were isolated and multiplied in liquid culture (PDA medium without agar). Dried colonies were then used for DNA extraction (Kårén *et al.* 1997). In 2006 and 2008, fungal sequences were obtained from DNA extracts of orchid roots, in order to reveal those fungi, which can not be cultured. The orchid roots were conserved in 96 % ethanol, after which DNA was extracted following the method of Kårén *et al.* (1997), except that the samples were ground in a Retsch MM200 mixer mill.

A polymerase chain reaction (PCR) was used for the amplification of the fungal nrITS region. The reactions were performed in a TECHNE TC312 thermal cycler, adopting the following program: 4.5 min/94 °C preliminary denaturation, followed by 33 three-step cycles of 30s/94 °C, 30s/51 °C and 45s/72 °C, then 7 min/72 °C final synthesis. In 2006 and 2008 multiple primer sets were used for the amplification of fungal DNA in a nested PCR reaction:

ITS1F–Tw13 and ITS1–ITS4 or ITS1F–Tw13 and ITS1-OF–ITS4-OF (White *et al.* 1990, Gardes & Bruns 1993, Taylor & McCormick 2008). In 2009 the primers ITS1 and ITS4 were used for the amplification. The PCR products were visualized on 1 % agarose gel containing 0.85 µg cm^{-3} ethidium bromide in an Alpha Multiimage Light Cabinet. The purified PCR products were submitted to dideoxy-cycle DNA sequencing with fluorescent terminators using a BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems) with the primers ITS1 and ITS4 or ITS1-OF and ITS4-OF. Capillary electrophoresis was carried out using an ABI PRISM 3100 Genetic Analyser (Applied Biosystems) according to the manufacturer's instructions.

All the DNA sequences were compared with those available in the GenBank nucleotide database (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) using BLAST (Zhang *et al.* 2000). The sequences were aligned with the MAFFT program (Katoh *et al.* 2005), and manually adjusted with the MEGA5 program package (Tamura *et al.* 2011). The sequences were converted into various file formats with ALTER (online free application, Glez-Peña *et al.* 2010). P distances between the obtained sequences were calculated with the PAUP program package, using the distance matrix option (Swofford 2003). Phylogenetic analysis, applying the ML criterion, was conducted with RaxML GUI 0.95 (Silvestro & Michalak 2011), using the rapid bootstrap option with a subsequent search for the best tree, employing the GTR+GAMMA+I model, and performing 1000 bootstrapping replicates.

The ITS sequences were deposited in the GenBank nucleotide database with the following accession numbers: AM711604, AM711609-AM711610, AM711613-AM711614, AM711618, AM711622, FR676937, HQ834811-HQ834845.

Soil analysis and coenological records

Besides collecting orchid individuals, soil analysis and a coenological survey was also performed in the three mines. One kg of soil was collected for each sample, after removing the litter and the covering vegetation. Two samples were collected in Pusztavám (in 2007 and 2009), six in Székesfehérvár (in 2007, 2008 and 2009) and three in Tokodaltáró (in 2008 and 2009). Soil samples were analysed at the Central Agricultural Office, Directorate of Plant Protection and Soil Conservation. Features of the soil samples were compared with Principal Component Analysis (PCA), computations were made by the SYNTAX5 package (Podani 1993).

Coenological records were taken in 2007. Vascular plants were identified in 2 × 2 m quadrates, then the degradation tolerance of the plants was determined according to the Hungarian Flora Database (Horváth *et al.* 1995). Simpson's diversity index was also calculated, then correlated (Pearson's correlation) with the number of the obtained fungal genera. Calculations were made with Microsoft Office Excel 2003.

Results

Identification of fungal endophytes

Altogether 42 sequences were obtained from the roots of the orchid plants, 24 of which are members of the form genus *Rhizoctonia* (Tabs. 1, 3), while the other 18 are various basidiomycetous and ascomycetous fungi (Tabs. 2, 4).

The sequences related to *Rhizoctonia* could be divided into four main clades according to the phylogenetic tree of the nrITS sequences (Fig. 1). The clades were identified based on reference sequences with known identities, chosen from the GenBank nucleotide database.

The Ceratobasidiaceae clade contains 11 sequences, all but one of which were identical sequences, obtained from *Orchis laxiflora* ssp. *palustris* plants in Székesfehérvár. These sequences showed 93-97 % similarity with various *Ceratobasidium* or *Rhizoctonia* sequences (EU668239 from Germany, AB454411 from Japan, AJ318420 from Singapore and GU206540 from Colombia). Sequence 9DI1-9A from the *Dactylorhiza incarnata* plant found in Tokodaltáró was 72 % identical with other sequences in this clade and it showed 93 % similarity with a *Rhizoctonia* sp. obtained from the tropical orchid *Tolumnia variegata* (AY443531 from Puerto Rico).

The Sebacinaceae clade contained only one sequence from an Epipactis *palustris* plant found in Tokodaltáró. It was 87-88 % similar to uncultured members of Sebacinales (HM451797 from Ecuador and FJ788825 from South Africa).

Two very different *Epulorhiza* clades could be seen on the phylogenetic tree. The *Epulorhiza 1* clade contained three sequences. The sequences obtained were 98 % identical to each other and all originated from an *Orchis laxiflora* ssp. *palustris* plant found in Székesfehérvár. The members of this group were 98 % similar to *Tulasnella calospora* (GU166415 from Thailand), 97–98 % similar to various *Epulorhiza* spp. (FJ613269 from China amd AJ313446 from Singapore) and 96 % similar to an uncultured Tulasnellaceae clone (GQ241783 from China).

The *Epulorhiza 2* clade contained nine sequences, which were obtained from *Orchis militaris* plants from Pusztavám and Tokodaltáró and from *Dactylorhiza incarnata* collected in Székesfehérvár. This heterogenous group of fungi could be divided into three subgroups, all of which contained almost identical sequences (96–99 % similarity), while the similarity between the subgroups was only 69–84 %. The first subgroup of the *Epulorhiza 2* clade, containing sequences 8DI2 and 6OMt76, was 96–99 % similar to an uncultured Tulasnellaceae mycorrhiza (AY634130 from Germany). The second subgroup, containing sequences 8OMt1 and 6DI2, was 98–99 % identical with an *Epulorhiza* sp. (AB369933 from Japan) and an uncultured member of Tulasnellaceae (GQ907269 from the Netherlands). The third subgroup contained five sequences, which were 99–100 % identical with uncultured sequences, all obtained from *O. militaris* across Europe (GQ907266 from Belgium, EU490419 from Italy and EU195344 from Estonia).

Tab. 1. Summary of the *Rhizoctonia*-fungi present in the roots of orchids investigated in the three mine wastes. The codes beginning with '9' represent isolated fungal strains, the codes beginning with '8' and '6' represent fungal clones.

Acc.No.	Code	Host orchid species	Habitat	Closest relative from GenBank and similarity %	Year of collection
HQ834818	9OL1-8A	*Orchis laxiflora* ssp. *palustris*	Székesfehérvár	AM697958 fungal sp. from *Liparis loeselii*, Hungary, 99 %	2009
HQ834819	9OL1-7A	*Orchis laxiflora* ssp. *palustris*	Székesfehérvár	AM697946 fungal sp. from the roots of *Dactylorhiza incarnata*, Hungary, 99 %	2009
HQ834820	9OL1-3A	*Orchis laxiflora* ssp. *palustris*	Székesfehérvár	AM697946 fungal sp. from the roots of *Dactylorhiza incarnata*, Hungary, 99 %	2009
HQ834824	9OL1-4A	*Orchis laxiflora* ssp. *palustris*	Székesfehérvár	AM697946 fungal sp. from the roots of *Dactylorhiza incarnata*, Hungary, 99 %	2009
HQ834816	9OL2-1A	*Orchis laxiflora* ssp. *palustris*	Székesfehérvár	AM697958 fungal sp. from *Liparis loeselii*, Hungary, 99 %	2009
HQ834817	9OL2-2A	*Orchis laxiflora* ssp. *palustris*	Székesfehérvár	AM697958 fungal sp. from *Liparis loeselii*, Hungary, 99 %	2009
HQ834822	9OL2-3B	*Orchis laxiflora* ssp. *palustris*	Székesfehérvár	AM697946 fungal sp. from the roots of *Dactylorhiza incarnata*, Hungary, 99 %	2009
HQ834825	9OL2-3C	*Orchis laxiflora* ssp. *palustris*	Székesfehérvár	AM697958 fungal sp. from *Liparis loeselii*, Hungary, 99 %	2009
HQ834827	9OL2-5A	*Orchis laxiflora* ssp. *palustris*	Székesfehérvár	AM697958 fungal sp. from *Liparis loeselii*, Hungary, 99 %	2009
HQ834831	9OL2-7A	*Orchis laxiflora* ssp. *palustris*	Székesfehérvár	AM697946 fungal sp. from the roots of *Dactylorhiza incarnata*, Hungary, 99 %	2009
HQ834828	9OL1-1C	*Orchis laxiflora* ssp. *palustris*	Székesfehérvár	FJ613269 Epulorhiza sp. from *Cymbidium faberi*, China, 98 %	2009
HQ834830	9OL1-8B	*Orchis laxiflora* ssp. *palustris*	Székesfehérvár	AJ549133 mycorrhizal fungus from the roots of *Orchis laxiflora* ssp. *palustris*, Hungary99 %	2009
HQ834836	9OL1-6A	*Orchis laxiflora* ssp. *palustris*	Székesfehérvár	FJ613269 Epulorhiza sp. from *Cymbidium faberi*, China, 97 %	2009

Acc.No.	Code	Host orchid species	Habitat	Closest relative from GenBank and similarity %	Year of collection
AM711609	6OMt41	*Orchis militaris*	Pusztavám	GQ907266 uncultured Tulasnellaceae clone from the roots of *Orchis militaris*, Belgium, 99 %	2006
AM711610	6OMt56	*Orchis militaris*	Pusztavám	GQ907266 uncultured Tulasnellaceae clone clone from the roots of *Orchis militaris*, Belgium, 99 %	2006
HQ834813	8OMt1	*Orchis militaris*	Pusztavám	AB369933 *Epulorhiza* sp. from the roots of *Cypripedium macranthos* var. *speciosum*, Japan, 99 %	2008
HQ834814	8OMt5-6	*Orchis militaris*	Pusztavám	AM711617 uncultured mycorrhizal fungus from the roots of *Epipactis palustris*, Hungary, 100 %	2008
AM711604	6OMt36	*Orchis militaris*	Tokodaltáró	AM711607 uncultured mycorrhizal fungus from the roots of *Epipactis palustris*, Hungary, 99 %	2006
AM711618	6OMt76	*Orchis militaris*	Tokodaltáró	AY634130 uncultured mycorrhiza (Tulasnellaceae) from the roots of *Dactylorhiza majalis*, Germany, 99 %	2006
AM711614	6DI2	*Dactylorhiza incarnata*	Tokodaltáró	GQ907269 uncultured Tulasnellaceae clone from the roots of *Orchis militaris*, the Netherlands, 99 %	2006
AM711613	6OMt4p	*Orchis militaris*	Tokodaltáró	GQ907266 uncultured Tulasnellaceae clone from the roots of *Orchis militaris*, Belgium, 100 %	2006
HQ834812	8DI2	*Dactylorhiza incarnata*	Székesfehérvár	AY634130 uncultured mycorrhiza (Tulasnellaceae) from the roots of *Dactylorhiza majalis*, Germany, 96 %	2008
AM711622	6EP5-11	*Epipactis palustris*	Tokodaltáró	FM177768 uncultured fungus from the roots of *Dactylorhiza incarnata*, Hungary, 98 %	2006
HQ834829	9DI1-9A	*Dactylorhiza incarnata*	Tokodaltáró	FJ820495 uncultured fungus clone from air sample, Germany, 99 %	2009

Tab. 2. Summary of the non-*Rhizoctonia* fungi present in the roots of orchids investigated in the three mine wastes. The codes beginning with '9' represent isolated fungal strains, the codes beginning with '8' represent fungal clones.

Acc.No.	Code	Host orchid species	Habitat	Closest relative from GenBank and similarity %	Year of collection
HQ834823	9OMt10-3A	*Orchis militaris*	Pusztavám	FJ478115 *Coprinopsis atramentaria* strain 98 %	2009
HQ834821	9OL2-3A	*Orchis laxiflora* ssp. *palustris*	Székesfehérvár	FJ478115 *Coprinopsis atramentaria* strain 97 %	2009
HQ834834	9OMt11-1A	*Orchis militaris*	Pusztavám	HM214456 *Fusarium solani* isolate from roots of *Cymbidium* sp., China, 100 %	2009
HQ834835	9OL1-1B	*Orchis laxiflora* ssp. *palustris*	Székesfehérvár	HM214456 *Fusarium solani* isolate from roots of *Cymbidium* sp., China, 100 %	2009
HQ834833	9OL2-6A	*Orchis laxiflora* ssp. *palustris*	Székesfehérvár	GU256752 *Fusarium oxysporum* strain 99 %	2009
HQ834837	9OMt12-1A	*Orchis militaris*	Székesfehérvár	HM214456 *Fusarium solani* isolate from roots of *Cymbidium* sp., China, 99 %	2009
HQ834840	9OMt12-1B	*Orchis militaris*	Székesfehérvár	HM132003 uncultured soil fungus clone from China, 99 %	2009
HQ834832	9OMt8-6A	*Orchis militaris*	Pusztavám	AY634148 uncultured mycorrhizal ascomycete from roots of *Epipactis atrorubens*, Germany, 99 %	2009
HQ834838	9DI1-3A	*Dactylorhiza incarnata*	Tokodaltáró	DQ182423 uncultured ascomycete isolate from roots of *Cephalanthera longifolia*, 99 %	2009
HQ834839	9DI1-7A	*Dactylorhiza incarnata*	Tokodaltáró	DQ182423 uncultured ascomycete isolate from roots of *Cephalanthera longifolia*, 99 %	2009
HQ834841	9DI1-5A	*Dactylorhiza incarnata*	Tokodaltáró	DQ182423 uncultured ascomycete isolate from roots of *Cephalanthera longifolia*, 99 %	2009
HQ834842	9DI1-5B	*Dactylorhiza incarnata*	Tokodaltáró	DQ182423 uncultured ascomycete isolate from roots of *Cephalanthera longifolia*, 99 %	2009
HQ834843	9DI1-6B	*Dactylorhiza incarnata*	Tokodaltáró	DQ182423 uncultured ascomycete isolate from roots of *Cephalanthera longifolia*, 99 %	2009

Acc.No.	Code	Host orchid species	Habitat	Closest relative from GenBank and similarity %	Year of collection
HQ834845	9DI1-7B	*Dactylorhiza incarnata*	Tokodaltáró	DQ182423 uncultured ascomycete isolate from roots of *Cephalanthera longifolia*, 99 %	2009
FR676937	8OC1b	*Orchis coriophora*	Székesfehérvár	GQ223455 uncultured Pezizales clone from roots of *Gymnadenia conopsea*, Germany, 88 %	2008
HQ834811	8OC1	*Orchis coriophora*	Székesfehérvár	GQ223455 uncultured Pezizales clone from roots of *Gymnadenia conopsea*, Germany, 88 %	2008
HQ834815	8OC2	*Orchis coriophora*	Székesfehérvár	GQ223455 uncultured Pezizales clone from roots of *Gymnadenia conopsea*, Germany, 91 %	2008
HQ834844	8OC2b	*Orchis coriophora*	Székesfehérvár	GQ223455 uncultured Pezizales clone from roots of *Gymnadenia conopsea*, Germany, 88 %	2008

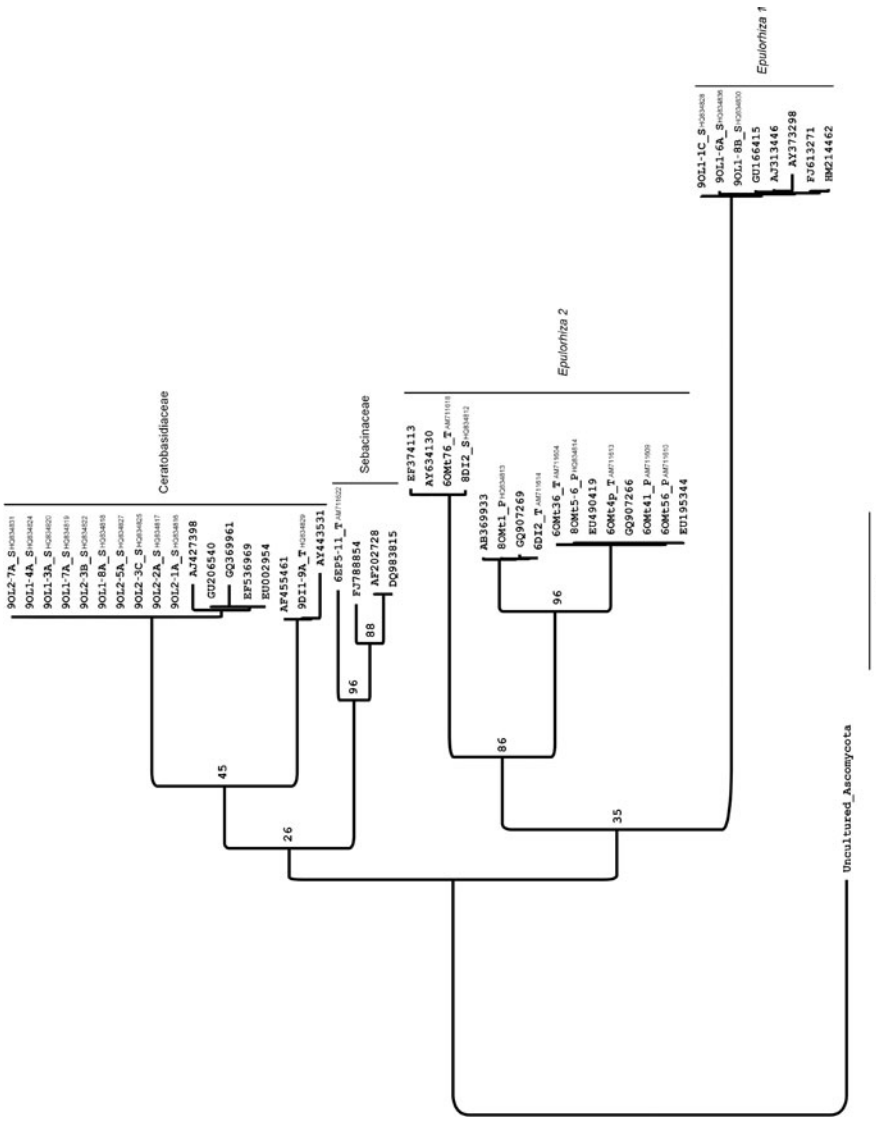

Fig. 1. Maximum-Likelihood consensus tree of the orchid mycorrhizal fungal sequences obtained and some reference sequences from GenBank. Bootstrap values (% of 1000 replications) are given. Scale bar indicates nucleotide changes per position. In the orchid mycorrhizal sequence codes the host orchid species and the habitat are abbreviated as follows: DI – *Dactylorhiza incarnata*, EP – *Epipactis palustris*, OL – *Orchis laxiflora* ssp. *palustris*, OMt – *Orchis militaris*; P – Pusztaván, S – Székesfehérvár, T – Tokodaltáró.

Tab. 3. p-distances between the obtained *Rhizoctonia*-sequences.

	8DI2_S	8OMt1_P	8OMt5-6_P	9OL2-1A_S	9OL2-2A_S	9OL1-8A_S	9OL1-7A_S	9OL1-3A_S	9OL2-3B_S	9OL1-4A_S	9OL2-3C_S
8DI2_S											
8OMt1_P	0,36955246										
8OMt5-6_P	0,41719872	0,17937042									
9OL2-1A_S	0,4223043	0,38882515	0,4080115								
9OL2-2A_S	0,4223043	0,38882515	0,4080115	0							
9OL1-8A_S	0,41865546	0,38569859	0,40813592	0	0						
9OL1-7A_S	0,41209459	0,38017827	0,40814027	0	0	0					
9OL1-3A_S	0,41209459	0,38017827	0,40814027	0	0	0	0				
9OL2-3B_S	0,41209459	0,38017827	0,40814027	0	0	0	0	0			
9OL1-4A_S	0,41209459	0,38017827	0,40814027	0	0	0	0	0	0		
9OL2-3C_S	0,43568295	0,40023962	0,40823561	0	0	0	0	0	0	0	
9OL2-5A_S	0,43757042	0,40180215	0,40842432	0	0	0	0	0	0	0	0
9OL1-1C_S	0,47846112	0,48352593	0,50286627	0,52177441	0,52177441	0,51714963	0,50902033	0,50902033	0,50902033	0,50902033	0,53244519
9DI1-9A_T	0,40220836	0,38490811	0,39638111	0,29696739	0,29696739	0,29448822	0,29004383	0,29004383	0,29004383	0,29004383	0,3059698
9OL1-8B_S	0,50547403	0,51580614	0,50485861	0,53718054	0,53718054	0,53739202	0,53763032	0,53763032	0,53763032	0,53763032	0,53744054
9OL2-7A_S	0,41209459	0,38017827	0,40814027	0	0	0	0	0	0	0	0
9OL1-6A_S	0,48127732	0,4841603	0,50059456	0,52616829	0,52616829	0,52149749	0,51331288	0,51331288	0,51331288	0,51331288	0,53690422
6OMt41_P	0,38922343	0,16970947	0,00181059	0,39940745	0,39940745	0,39623606	0,39054257	0,39054257	0,39054257	0,39054257	0,40955055
6OMt56_P	0,3892647	0,1685176	0,00181242	0,39873582	0,39873582	0,39558333	0,38992822	0,38992822	0,38992822	0,38992822	0,40883279
6OMt36_T	0,3895857	0,16820103	0,00547804	0,39903373	0,39903373	0,39590296	0,39027417	0,39027417	0,39027417	0,39027417	0,40917304
6OMt76_T	0,00805838	0,36216339	0,41447124	0,41793507	0,41793507	0,41440335	0,40806901	0,40806901	0,40806901	0,40806901	0,43077287
6DI2_T	0,37056905	0,00464396	0,17491956	0,38855392	0,38855392	0,38545594	0,3799808	0,3799808	0,3799808	0,3799808	0,39992854
6EP5-11_T	0,39663687	0,38126373	0,39899457	0,35813564	0,35813564	0,35526466	0,35525295	0,35525295	0,35525295	0,35525295	0,36762023
6OMt4p_T	0,4148652	0,18042171	0,00183843	0,41442949	0,41442949	0,41457972	0,41472611	0,41472611	0,41472611	0,41472611	0,41464096

The non-*Rhizoctonia* sequences could also be divided in four clades, which were identified based on reference sequences with known identities from the GenBank nucleotide database.

The Pezizales group contained four sequences originating from an *Orchis coriophora* plant found in Székesfehérvár and exhibited 98-100 % similarity. The sequences were 88-91 % identical with an uncultured Pezizales clone from *Gymnadenia conopsea* (GQ223455 from Germany).

The Coprinaceae group contained two sequences, one from an *Orchis militaris* plant from Pusztavám, and the other from an *O. laxiflora* ssp. *palustris* plant from Székesfehérvár. These sequences were 99 % identical with each other. They were 97-98 % identical with a *Coprinopsis atramentaria*

	9OL2-5A_S	9OL1-1C_S	9DI1-9A_T	9OL1-8B_S	9OL2-7A_S	9OL1-6A_S	6OMt41_P	6OMt56_P	6OMt36_T	6OMt76_T	6DI2_T	6EP5-11_T	6OMt4p_T
	0,53480893												
	0,30732879	0,48072642											
	0,53771865	0,00364083	0,50225693										
	0	0,50902033	0,29004383	0,53763032									
	0,53927338	0,00839189	0,48412269	0,00367604	0,51331288								
	0,41125035	0,48957676	0,38045609	0,5121572	0,39054257	0,48996854							
	0,41052014	0,48986036	0,3805677	0,51256663	0,38992822	0,49025476	0						
	0,41088504	0,4938466	0,37999493	0,51706725	0,39027417	0,49425828	0,00310078	0,00310078					
	0,43260461	0,47889587	0,39863503	0,50520664	0,40806901	0,48153418	0,38725445	0,38734007	0,38887703				
	0,40150967	0,48449984	0,38803411	0,51679814	0,3799808	0,48513705	0,16619213	0,16498873	0,16469176	0,36317822			
	0,3693077	0,51418251	0,35671872	0,53413552	0,35525295	0,51933539	0,37897786	0,3791886	0,38230795	0,39165598	0,38347018		
	0,4148345	0,51538223	0,4032636	0,51207286	0,41472611	0,51580673	0	0	0,00330779	0,4124217	0,17660527	0,40178448	

strain in the GenBank nucleotide database (FJ478115 from China) and 93-94 % similar to a mycorrhizal basidiomycete from the roots of the achlorophyllous orchid *Epipogium roseum* (AB176577 from Japan).

The *Fusarium* group could be divided into two subgroups, one containing two sequences which were almost identical to each other (99 % similarity) and were obtained in Székesfehérvár from the roots of one *Orchis militaris* and one *O. laxiflora* ssp. *palustris* plant. These sequences were 99 % identical with different *Fusarium oxysporum* isolates (GU256752 from the USA, GU109337 from Poland). There were no sequence matches obtained from orchids in the closest 100 BLAST results for these isolates. The other subgroup contained three sequences, from one *O. militaris* and one *O. laxi-*

Tab. 4. p-distances between the obtained non-*Rhizoctonia*-sequences.

	8OC1b_S	8OC1_S	8OC2_S	9OL2-3A_S	9OMt10-3A_P	9OMt8-6A_P	9OL2-6A_S	9OMt11-1A_P
8OC1b_S								
8OC1_S	0							
8OC2_S	0,0214677	0,02143186						
9OL2-3A_S	0,38695887	0,38807595	0,37402132					
9OMt10-3A_P	0,39601427	0,39714041	0,37458614	0,00915979				
9OMt8-6A_P	0,35625869	0,35749984	0,34556398	0,38792601	0,40101516			
9OL2-6A_S	0,39827046	0,39941692	0,36797935	0,383517	0,39500394	0,24252781		
9OMt11-1A_P	0,39678633	0,39807919	0,35391346	0,36985734	0,38221818	0,28615212	0,20813583	
9OL1-1B_S	0,40934786	0,41062051	0,35720807	0,38184372	0,38230261	0,29791182	0,21354386	0,00755574
9OMt12-1A_S	0,40855035	0,40982711	0,35439721	0,38118017	0,37318864	0,29738414	0,21704826	0,00752851
9DI1-3A_T	0,36961627	0,37083688	0,35635951	0,40018031	0,4122051	0,04196228	0,24185066	0,28715274
9DI1-7A_T	0,36961627	0,37083688	0,35635951	0,40018031	0,4122051	0,04196228	0,24185066	0,28715274
9OMt12-1B_S	0,39890918	0,40004551	0,36734894	0,38870195	0,39705482	0,23870121	0,00193277	0,20758377
9DI1-5A_T	0,36961627	0,37083688	0,35635951	0,40018031	0,4122051	0,04196228	0,24185066	0,28715274
9DI1-5B_T	0,37119085	0,37241146	0,35651144	0,4016642	0,41218239	0,04210146	0,24226658	0,28819397
9DI1-6B_T	0,38163859	0,38285375	0,35687944	0,4123846	0,40352926	0,05084313	0,2526966	0,30015096
8OC2b_S	0	0	0,0214736	0,38610756	0,39512148	0,35555667	0,3996014	0,39809483
9DI1-7B_T	0,39536417	0,39671433	0,35740104	0,41551	0,41660514	0,04120127	0,26011825	0,31150627

flora ssp. *palustris* plant found in Székesfehérvár, and from one *O. militaris* plant collected in Pusztavám. They were 99–100 % identical with a *Fusarium solani* isolate from *Cymbidium* sp. (HM214456 from China). The sequences in each subgroup were 99–100 % similar to each other, while the similarity between the subgroups was 84–87 %.

The *Leptodontidium* group contained seven sequences, one of which was obtained from an *Orchis militaris* plant from Pusztavám, while the others originated from a *Dactylorhiza incarnata* plant from Tokodaltáró. All but one of the sequences were almost identical (99–100 % similarity). The similarity between the isolates in this clade was 95–96 %. Sequence 9OMt8-6A was similar to an uncultured mycorrhizal ascomycete obtained from *Epipactis atrorubens* (AY634148 from Germany), the other six isolates showed 99 % similarity with an uncultured ascomycete isolate from *Cephalanthera longifolia* (DQ182423 from France) and 98 % similarity with fungi from *Epipactis helleborine*, *Pterostylis nutans* and *Gymnadenia conopsea* (AY634168 from the USA, EF090490 from Australia and GQ223464 from Germany). According to BLAST searches these ascomycetous fungi belong to dark septate endophytes, possibly members of *Leptodontidium* spp. and *Cadophora* spp.

9OL1-1B_S	9OMt12-1A_S	9DI1-3A_T	9DI1-7A_T	9OMt12-1B_S	9DI1-5A_T	9DI1-5B_T	9DI1-6B_T	8OC2b_S	9DI1-7B_T
0,00756144									
0,29949006	0,2989704								
0,29949006	0,2989704	0							
0,21522644	0,21875253	0,23739237	0,23739237						
0,29949006	0,2989704	0	0	0,23739237					
0,29946801	0,29895017	0	0	0,23834234	0				
0,29962623	0,29078799	0,0084689	0,0084689	0,2502892	0,0084689	0,00846154			
0,41062528	0,40983188	0,36891767	0,36891767	0,40016347	0,36891767	0,37049225	0,380923		
0,31398761	0,31161115	0	0	0,25792912	0	0	0	0,39452323	

Soil analysis and coenological records

The PCA showed that the soil samples obtained in Pusztavám stand apart from the samples obtained in Székesfehérvár and Tokodaltáró (Fig. 2). The main factors causing the differences are the soil water-capacity (sticky point according to Arany: it gives the volume (ml) of the water which is taken up by 100 g dried soil; it is the highest in Tokodaltáró), humus- (highest in Székesfehérvár) and $CaCO_3$-content (highest in Pusztavám) of the soils.

According to the coenological survey, the most degraded habitat was in Pusztavám (Fig. 3), where most plant species tolerate well the degradation of the habitat. In Tokodaltáró, the majority of the abundant species tolerates well or moderately the degradation. The least degradation-tolerant plant community was found in Székesfehérvár, where the majority of plant species tolerates moderately or slightly the degradation of the habitat. The correlation between Simpson's diversity and the number of fungal genera obtained was strong (r = 0.54) when all the obtained fungal genera were taken into consideration, and very strong (r = 0.78) with respect only for the *Rhizoctonia*-genera.

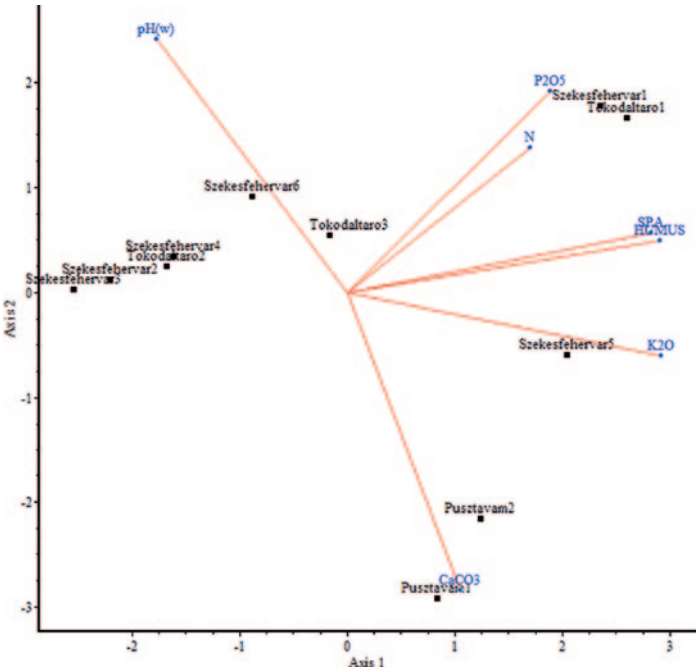

Fig. 2. PCA ordination diagram of the soil samples collected in the three mines and soil features represented with squares and dots respectively. Soil features are abbreviated as follows: pH(w) – pH measured in water; P2O5 – P_2O_5 content; N – NO_2+NO_3-N content; SPA – sticky point according to Arany; HUMUS – humus content; K2O – K_2O content; CaCO3 – $CaCO_3$ content.

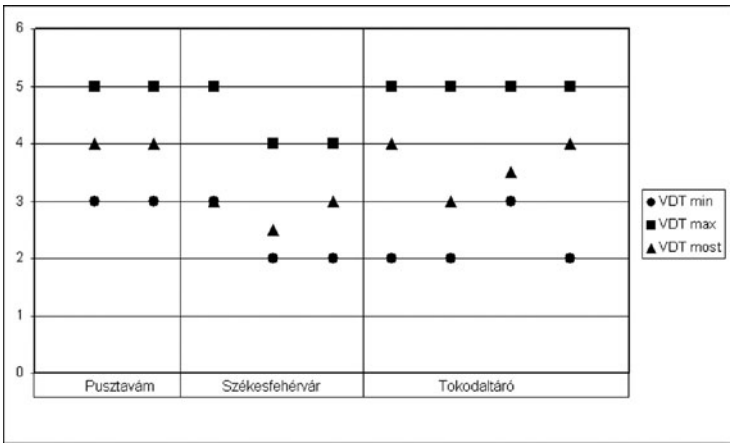

Fig. 3. Degradation tolerance values of the vascular plants in the three investigated mines. Abbreviations are as follows: VDT min – the minimum value of degradation tolerance; VDT max – the maximum value of degradation tolerance; VDT most – the most frequent degradation tolerance value.

Discussion

A very diverse community of fungi was obtained from orchid roots in the three investigated abandoned mines, comprising three fungal genera generally reported as orchid symbionts and four other basidiomycetous or ascomycetous fungal groups. The obtained fungi seem to be distributed worldwide, as the most similar sequences in the GenBank nucleotide database originate from various tropical and terrestrial (temperate zone) orchids, except for the *Fusarium oxysporum* isolates.

The most diverse fungal community was found in Székesfehérvár, where three groups of *Rhizoctonia* symbionts and three groups of non-*Rhizoctonia* endophytes were found. In Tokodaltáró the community was slightly less diverse but also diversified (three groups of classical symbionts and one ascomycetous endophyte group), while in Pusztavám it was restricted to only one group of *Rhizoctonia* symbionts and three groups of non-*Rhizoctonia* fungi. This may be related to the vegetation of the habitats, as in accordance to the correlation-tests, those with a more diverse and less disturbed flora had a more diverse symbiont community. It is important to note that coal was mined in Pusztavám and sand in the other two mines, which may also have caused differences in the composition of the fungal community. Nevertheless the present observations are in agreement with earlier studies (Waldrop *et al*. 2006) where plant and fungal diversity were found to be related to each other, further supporting the suggestion that differences between the fungal communities of mines are caused mainly by differences in the plant community. However, it is very difficult to distinguish clearly between the effects of the plant community and the mother rock on the composition of fungal communities since these are inter-related.

Isolates belonging to the Ceratobasidiaceae family and *Epulorhiza* seem to be common not only in Hungary, but also in other parts of Europe and in the tropical areas (Ma *et al*. 2003, Ogura-Tsujita *et al*. 2009, Waterman *et al*. 2011). In Hungary, sequences considered to belong to the Sebacinaceae were only obtained in small numbers. Other Hungarian studies (Illyés *et al*. 2009) also reported two different clades of fungi belonging to the *Epulorhiza*, but with different proportions, as the members of the *Epulorhiza 2* clade were three times more abundant in mine wastes than fungi belonging to the *Epulorhiza 1* clade. These results add support to the theory that *Epulorhiza* species may be present mostly in disturbed habitats (Bonnardeaux *et al*. 2007). It appears that this group of fungi is tolerant to unfavourable environmental circumstances, making it suitable for the colonisation of pioneer or disturbed locations such as mine wastes. This hypothesis is well supported by the fact that only *Epulorhiza* symbionts were found in Pusztavám, where the whole vegetation is in a pioneer state. Observations showed that the orchid mycorrhizal fungal community became more complex as secondary succession advanced and the vegetation approached that observed in natural habitats, though this contrasts with reports by Shefferson *et al*. (2008). In the mines in

Tokodaltáró and Székesfehérvár orchid mycorrhizal fungal genera other than *Epulorhiza* were also present (e.g. *Sebacina*, *Ceratobasidium*), as is observed in natural habitats in Hungary (Illyés *et al.* 2009). The mine sites in Tokodaltáró and Székesfehérvár seem to resemble natural habitats regarding not only with respect to the vegetation but also the orchid mycorrhizal fungi found there, especially in Székesfehérvár, where several members of the *Ceratobasidium* spp. and *Epulorhiza* spp. were detected.

The present work provides a basis for further discussion of the mycorrhizal specificity of *Orchis militaris*. The mycorrhizal specificity of *O. militaris* might be looser than suggested by Vendramin *et al.* (2010), as two other subgroups of *Epulorhiza* fungi and three non-*Rhizoctonia* groups were also obtained besides the two narrow Tulasnellaceae clades demonstrated by these authors. Moreover, other Hungarian studies (Illyés *et al.* 2009, Ouanphanivanh *et al.* 2007) detected fungi belonging to the Ceratobasidiaceae, Sebacinaceae and *Epulorhiza 1* from the roots of *O. militaris*. Taken together, it appears that *O. militaris* is a generalist in terms of the fungal specificity, but in mine wastes the orchid-fungus relationship becomes more specialised, as this is more convenient and effective under unfavourable environmental circumstances. It could have a positive effect on the seedling establishment and dispersal of *O. militaris*, as the distribution of this orchid is only slightly limited by the distribution of the appropriate fungal partner. Furthermore, the generalist strategy of *O. militaris* may reduce the intraspecific competition in a habitat, where various orchid mycorrhizal fungi are present.

To the best of our knowledge, this is the first report of the isolation of Coprinaceae from the roots of photosynthesising orchids such as *O. laxiflora* ssp. *palustris* and *O. militaris* and lends further support to the suggestion that members of the Coprinaceae could be in a mycorrhizal relationship with orchids (Yamato *et al.* 2005).

The role of *Fusarium* species in plants is very contradictory; some are considered as phytopathogens, but other *Fusarium* species may assist plants to tolerate heavy metal contamination (Rautaray *et al.* 2004, Sanyal *et al.* 2005) and their ability to induce orchid seed germination has also been reported (Vujanovic *et al.* 2000). Gezgin & Eltem (2009) isolated mainly *Fusarium* species (94 % of all isolates) from various Aegean and Mediterranean orchids, which supports the view that these fungi could be appropriate candidates for symbiotic seed germination experiments. The present observation that two of the *Fusarium* isolates were 99 % identical to *F. oxysporum* strains isolated from mine wastes in Mexico (Ortega-Larrocea *et al.* 2010) suggests that *Fusarium* species could play an advantageous role in the survival of orchids under such unfavourable conditions, either by aiding the toleration of contaminants or by stimulating seed germination.

There was no clear evidence whether the ascomycetous isolates obtained were mycorrhizal, pathogenic or parasitic, however dark septate endophytes have several characteristics similar to those of mycorrhizal fungi (i.e. im-

proved nutrient uptake and host growth), suggesting that they are mutualistic (Jumpponen 2001). It is possible that they have an important ecological role, either by forming mycelial connections between plants or by taking part in nutrient and water uptake when mycorrhizal fungi are inhibited by the environmental conditions (Jumpponen & Trappe 1998). It might be very advantageous for orchids to co-exist with such fungi, especially in mines.

The fact that various non-*Rhizoctonia* orchid endophytes were also found raises the possibility that these fungi could play an important role in helping orchids to tolerate the unfavourable circumstances in mine wastes.

These results may cast some further light on the fungal specificity of orchids, particularly of *O. militaris*. Evidence is emerging that the habitat requirements of orchid mycorrhizal fungi are factors as relevant to the specificity of orchid-fungal interactions as the habitat requirements of orchids. Future work should be devoted to determining the environmental demands of orchid mycorrhizal fungi to provide useful information for orchid conservation efforts.

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