Mycorrhizal interactions of orchids colonizing Estonian mine tailings hills

Richard P. Shefferson,1,2,4 Tiu Kull,3 and Kadri Tali3

1Microbial Ecology Laboratory, Forestry and Forest Products Research Institute, 1 Matsunosato, Tsukuba 305-8687 Japan; and
2Institute of Agricultural and Environmental Sciences, Riia 181, Tartu 51014 Estonia

Northeastern Estonia is home to extensive oil shale mines. Associated with these are desolate and environmentally damaging hills of ash and semicoke tailings. Interestingly, some of the first plants to colonize these hills are rare orchids. Here, we assess the identities of the mycorrhizal fungi associated with these orchids, in particular Epipactis atrorubens, Orchis militaris, and Dactylorhiza baltica, and compare them with mycorrhizal fungi from orchids from pristine habitat. Epipactis atrorubens was associated with the widest breadth of fungi, including unnamed members of the basidiomycete family Tulasnellaceae and the potentially ectomycorrhizal ascomycetes Trichophaea woolhopea and Geopora cooperi. Orchis militaris also associated with unnamed members of the Tulasnellaceae. Dactylorhiza baltica associated with Ceratobasidium albastiensis. In Epipactis and Orchis, the same fungi associated with plants in the pristine habitat as with those on ash hills. The tulasnelloid and ceratobasidioid fungi mycorrhizal with these orchids appear closely related to common orchid mycorrhizal fungi, while one of the ascomycetes mycorrhizal with E. atrorubens is closely related to a mycorrhizal fungus with E. microphilla. Our results suggest that these orchids and their fungi are not limited to pristine habitats and that environmentally polluted sites may present novel habitats that may be exploited for endangered plant conservation.

Key words: Ceratobasidium; colonization; mine tailings; mycorrhizal ascomycete; orchid conservation; primary succession; Tulasnellaceae.

Orchids are well known for their rarity, but are also increasingly well known for their specialization to particular habitats (Linder, 1995), pollinators (Darwin, 1862; Cozzolino and Widmer, 2005), and mycorrhizal associates (Taylor and Bruns, 1997; McCormick et al., 2004; Shefferson et al., 2007). Such specialization may contribute to both the diversity and the rarity of orchid species (Gill, 1989; Shefferson et al., 2005; Otero and Flanagan, 2006). Because declining geographic ranges and population extinctions have been widely noted throughout the orchid family and across habitat types (Kull and Hutchings, 2006), specialization may increase the chance of extinction for those orchids most specialized to endangered or rare habitats.

Estonia is home to some of the world’s most heavily mined deposits of oil shale, a sedimentary rock with large deposits of organic matter (Trinnaman and Clarke, 2004). This rock provides for the bulk of Estonia’s electricity production as well as some industrial chemicals (Öpik, 1989; Raukas, 2004). Since commercial mining began in 1916, hills of semicoke and ash tailings from oil shale mines have accumulated throughout northeastern Estonia, particularly around the city of Narva (Toomik and Liblik, 1998; Brendow, 2002). Environmental degradation resulting from mine tailings hills includes sulfur and toxic metal contamination of soils and groundwater and the deterioration and destruction of forests (United Nations Economic Commission for Europe, 2001; Teinemaa et al., 2002).

Both semicoke and ash also prove inhospitable to most life, at least in the near term. Nonetheless, life eventually colonizes these hills, and surprisingly, some of the first plants to colonize these tailings hills are orchids. Such colonization trends are surprising exceptions to the prevailing tendency toward specialization in the Orchidaceae and suggest greater tolerance to habitat conditions than previously assumed.

In the wild, orchid seeds germinate only upon being colonized by an appropriate mycorrhizal fungus (Rasmussen, 1995). Development to maturity generally requires the absorption of carbon provided by the fungal partner (Rasmussen, 1995; Smith and Read, 1997), with some orchids continuing to live on fungal carbon in maturity as well, forsaking the production of sugars via photosynthesis altogether (Leake, 1994; Taylor and Bruns, 1997). Thus, orchids are particularly dependent on their mycorrhizal partners for much of their nutrition. In this study, we asked whether orchids occurring on mine tailings sites developed mycorrhizae and assessed the identities of those fungi present. We further asked if the identities of the mycorrhizal fungi varied across habitats. We used this knowledge to explore the ecology of orchid mycorrhizal fungi in the context of primary succession and conservation.

MATERIALS AND METHODS

We sampled a total of nine individuals of three orchid species on and off a mine tailing habitats in northeastern Estonia in July 2005. Epipactis atrorubens (Hoffm.) Bess. is a short-statured, rhizomatous orchid occurring on calcareous soils throughout Europe. One individual was sampled from the youngest tailings hill in the study (Site 5, Table 1; Fig. 1), and so were two individuals from pristine habitat on the islands of Muhumaa and Hiiumaa (sites 4 and 6, respectively, Table 1). Orchis militaris L. is a pseudobulbous orchid growing a 20–50 cm long inflorescence. Three individuals were sampled at a working oil shale mine in the older sections of a tailings hill (site 1, Table 1). One further individual was sampled in a 200-yr-old forest on the island of Hiiumaa (site 2, Table 1). Dactylorhiza baltica (Klinge) Nevska is a rare orchid occurring primarily in eastern Germany and eastward into Russia (Shipunov et al., 2005). Two individuals were sampled from a tailings site onto which Populus spp. had been
planted to provide a sparse woodland habitat (site 3, Table 1). This species was not sampled under pristine conditions due to its rarity.

Whole individuals were taken to assess mycorrhizal colonization in the entire root system. Numbers of individuals sampled per species were kept low for conservation concerns and permit requirements. Roots were sectioned at 1-cm intervals throughout the entire root system, and a compound microscope was used to scan sections for mycorrhizal hyphal coils called pelotons growing intracellularly within root cortical cells. These pelotons are the primary evidence of the establishment of the orchid mycorrhiza (Rasmussen, 1995). Percentage colonization was assessed as the proportion of sections containing pelotons to the total number of sections per plant.

A roughly 1-cm-long chunk of root tissue corresponding to each cross section that had been viewed under the microscope was used as substrate for DNA extraction, with a range of 1 – 6 samples per plant, and a total of 30 DNA samples. Only the DNA of mycorrhizally colonized samples was extracted. DNA extraction proceeded via the DNeasy Plant Mini Kit (Qiagen, Valencia, California, USA). We attempted to PCR amplify the fungal nuclear internal transcribed spacer (hereafter, ITS) and nuclear 5.8S rDNA regions using the primer sets ITS1F-ITS4 (White et al., 1990; Gardes and Bruns, 1993) and ITS1OF-ITS4OF (developed by D. L. Taylor and available online at http://mercury.bio.uaf.edu/~lee_taylor/PCR_Primers_Orchid_Fungi.html). We also attempted to PCR amplify the mitochondrial large subunit rDNA region (hereafter, mtLSU) using the primer set ML5-ML6 (Bruns et al., 1998). Multiple primer sets were used to find as many root endophytes as possible and to account for the potential for bias from potentially differing densities of tissue among fungi co-occurring within the same plant roots. PCR proceeded with 35 cycles and an annealing temperature of 55°C on an ABI 9700 Thermocycler (Applied Biosystems, Foster City, California, USA).

Mycorrhizal diversity across samples was assessed via three-enzyme RFLP analysis of ITS1F-ITS4 primer pair-based PCR products. ITS-RFLPs were assessed for Dactylorhiza baltica and Epipactis atrorubens with enzymes HinfI, MspI, and NlaIII. ITS-RFLPs were assessed for Orchis militaris using enzymes DdeI, DpnI, and HinfI (Table 2). Additionally, we confirmed our ITS-RFLP results using two-enzyme RFLP analysis on PCR products from primer pairs ITS1OF-ITS4OF and ML5-ML6, using enzymes MspI and NlaIII. At least one sample from each unique RFLP type was chosen as representative of the diversity of mycorrhizal fungi across sections and study individuals, and the ITS1F-ITS4 PCR products of these samples were subject to DNA sequencing using ABI BigDye 3.1 chemistry and an ABI 3100 16-capillary DNA sequencer (Applied Biosystems, Foster City, California, USA). Where PCR results differed

### Table 1. Locations and descriptions of study sites. All sites were located in the Baltic coast nation of Estonia. Asterisks denote alvar habitat, which occurs on limestone with thin or no soil, thus leading to sparse vegetation.

<table>
<thead>
<tr>
<th>Pop. no.</th>
<th>Location</th>
<th>Species sampled</th>
<th>Description</th>
<th>Latitude</th>
<th>Longitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Johvi-Kose</td>
<td>Orchis militaris</td>
<td>10–20-yr-old semicoke hill with Hippophae rhamnoides and planted Populus trees</td>
<td>59°23’29.5&quot;N</td>
<td>27°13’48.8&quot;E</td>
</tr>
<tr>
<td>2</td>
<td>Hiiumaa</td>
<td>O. militaris</td>
<td>200-yr-old alvar* forest dominated by Pinus spp.</td>
<td>58°58’43.1&quot;N</td>
<td>22°29’35.7&quot;E</td>
</tr>
<tr>
<td>3</td>
<td>Johvi-Kose</td>
<td>Dactylorhiza baltica</td>
<td>30–40-yr-old ash hill with planted Populus trees</td>
<td>59°19’10.5&quot;N</td>
<td>27°28’10.4&quot;E</td>
</tr>
<tr>
<td>4</td>
<td>Muhumaa</td>
<td>Epipactis atrorubens</td>
<td>Alvar* meadow on coastal farm site</td>
<td>58°40’06.5&quot;N</td>
<td>23°14’56.7&quot;E</td>
</tr>
<tr>
<td>5</td>
<td>Johvi-Kose</td>
<td>E. atrorubens</td>
<td>Ash hill currently being constructed, with carpet of E. atrorubens and scattered Populus saplings</td>
<td>59°19’38.1&quot;N</td>
<td>27°28’33.1&quot;E</td>
</tr>
<tr>
<td>6</td>
<td>Hiiumaa</td>
<td>E. atrorubens</td>
<td>Roadside population by coast and pine forest</td>
<td>58°51’25.1&quot;N</td>
<td>23°02’49.2&quot;E</td>
</tr>
</tbody>
</table>

![Fig. 1](image-url) Oil shale ash hills at Johvi-Kose in the vicinity of Narva, Estonia (site 5, Table 1). Amateur botany group is exploring one ash hill, with planted poplar saplings visible in the foreground and further ash hills, electricity plants, and forests visible in the background.
among primer sets, we also sequenced representative samples with one or both of the other primers.

We used NCBI BLAST to assess the most likely fungal families that study sequences corresponded with. Then, study sequences were aligned with sequences of reference taxa in CLUSTAL_X v.1.81 (Thompson et al., 1997), and unalignable regions were removed. In the case of the Tulasnellaceae ITS data, this resulted in alignments of the 5.8S rDNA because the ITS1 and ITS2 regions were too divergent to be aligned (Weiß and Oberwinkler, 2001; Taylor et al., 2003; Suárez et al., 2006). MODELTEST 3.7 was used to determine the most appropriate models of nucleotide evolution (Posada and Crandall, 1998; Posada and Buckley, 2004). Phylogenetic analysis proceeded via maximum likelihood analysis in PHYML 2.4 for Windows (Guindon and Gascuel, 2003; Guindon et al., 2005). For each alignment, the model of nucleotide substitution was set to the model recommended by MODELTEST, as were the proportion of invariable sites and the gamma rate parameter. We further tested the robustness of our phylogenies with 500 parametric bootstrap replicates via PHYML 2.4 (Guindon and Gascuel, 2003; Guindon et al., 2005). Phylogenetic analysis suggested that mycorrhizal interaction did not vary with habitat type. Mycorrhizal fungi found in individuals growing on ash hills were generally the same species as those found in individuals growing in pristine sites (Table 2). Epipactis atrorubens associated with the broadest range of fungi. The individual on the pristine habitat in Hiiumaa associated exclusively with an unnamed member of the basidiomycete family Tulasnellaceae (Table 2; Fig. 3). In contrast, the other individual from the pristine habitat as well as the individual from mine tailings habitat associated with ascomycetes: the individual from Muhumaa associated with a potentially ectomycorrhizal fungus, which may be Trichophaea woolhoepia, while the individual from the mine tailings associated with Geopora cooperi (Table 2; Fig. 4). The latter individual also included one DNA sample containing an ITS product from Chalara dualis (Table 2; Fig. 4). All Orchis militaris individuals, both from mine tailings hills and pristine habitats, associated with an unnamed member of the Tulasnellaceae (Table 2; Figs. 3, 5). Dactylorhiza baltica associated exclusively with Ceratobasidium albasitensis (Fig. 6).

Phylogenetic analysis suggested that mycorrhizal interaction did not vary with habitat type. Mycorrhizal fungi found in individuals growing on ash hills were generally the same species as those found in individuals growing in pristine sites (Table 2). Epipactis atrorubens associated with the broadest range of fungi. The individual on the pristine habitat in Hiiumaa associated exclusively with an unnamed member of the basidiomycete family Tulasnellaceae (Table 2; Fig. 3). In contrast, the other individual from the pristine habitat as well as the individual from mine tailings habitat associated with ascomycetes: the individual from Muhumaa associated with a potentially ectomycorrhizal fungus, which may be Trichophaea woolhoepia, while the individual from the mine tailings associated with Geopora cooperi (Table 2; Fig. 4). The latter individual also included one DNA sample containing an ITS product from Chalara dualis (Table 2; Fig. 4). All Orchis militaris individuals, both from mine tailings hills and pristine habitats, associated with an unnamed member of the Tulasnellaceae (Table 2; Figs. 3, 5). Dactylorhiza baltica associated exclusively with Ceratobasidium albasitensis (Fig. 6).

RESULTS

All individuals had evidence of root colonization by mycorrhizal fungi (Fig. 2). The root systems of Epipactis atrorubens and Orchis militaris were 100% colonized, with pellets in at least some cortical cells in each root section (Fig. 2). In Dactylorhiza baltica, one individual was 100% colonized, while the other was only 50% colonized. Primer pair ITS1F-ITS4 yielded PCR product in all samples, while primer pairs ITS1OF-ITS4OF and ML5-ML6 yielded PCR product in 50% and 30% of samples, respectively (Table 2).

Initial BLAST searches indicated that the study species associated with fungi in the Tulasnellaceae, Ceratobasidiaceae, and Pezizales. MODELTEST suggested the general time-reversible model of nucleotide evolution with rate heterogeneity across sites (GTR + I) for our ITS Tulasnellaceae (Γ = 0.419) and ITS Ceratobasidiaceae (Γ = 0.328) alignments (Tavaré, 1986), the general time-reversible model with a proportion of sites invariant and rate heterogeneity across sites (GTR + I + Γ; I = 0.272 and Γ = 1.610) for our ITS Pezizales alignment (Tavaré, 1986), and the Hasegawa-Kishino-Yano model with a proportion of sites invariant and rate heterogeneity across sites (HKY + I + Γ; I = 0.321 and Γ = 0.972) for our nmtLSU alignment (Hasegawa et al., 1985).

Fig. 2. Cross sections of an Orchis militaris root showing fungal pellets within root cortical cells. Scale bar = 25 μm.
DISCUSSION

Although the orchids appeared to be fairly specialized to particular fungi in this study, they did not appear limited geographically by these specific interactions. Mycorrhizal fungal species associate with these orchids across a wide range of habitats, including both heavily polluted and relatively pristine sites. These sites represent radically different nutrient conditions and may also have different hydrologies and daily temperature ranges. Although we could not sample substantial numbers of these species due to conservation concerns, our results suggest that mycorrhizal specificity in these orchids may not be wide
because we did not find much variation in comparisons of individuals of the same species growing on different sites. Similar results have been reported by Shefferson et al. (2005, 2007); the most and least widespread Cypripedium species, *C. calceolus* and *C. californicum*, respectively, were among the most and least mycorrhizally specialized, respectively. Our results also add support to the notion that photosynthetic orchids may run the range of specificity from fairly generalist to highly specialized.
Fig. 5. Fungal mitochondrial large subunit (mtLSU) rDNA phylogeny showing that fungi mycorrhizal with *Orchis militaris* are orchid mycorrhizal members of the Tulasnellaceae. Phylogeny constructed with 227 bp and 36 taxa, and rooted with *Gomphus floccosus* and *Ramaria araiospora*. The best tree resulting from a heuristic maximum likelihood search in PHYML is presented, with support values derived from 500 bootstrap replicates. Values at nodes indicate percentage bootstrap support.
mycorrhizal fungi (Roberts, 1999; Selosse et al., 2004) and generally agree with fungal groups noted as mycorrhizal with other members of these respective orchid genera (Rasmussen, 1995). The conditions on mine tailings hills may represent ecologically novel niches, and the lack of other established plants and (Otero et al., 2002; McCormick et al., 2004; Otero et al., 2004) and that orchid mycorrhizal interactions may persist as highly specialized in times of environmental stress for the plant (McCormick et al., 2006). Phylogenetically, the fungal species identified in this study run the wide, polyphyletic breadth of orchid mycorrhizal fungi (Roberts, 1999; Selosse et al., 2004) and generally agree with fungal groups noted as mycorrhizal with other members of these respective orchid genera (Rasmussen, 1995). The conditions on mine tailings hills may represent ecologically novel niches, and the lack of other established plants and

---

**Fig. 6.** Fungal ITS + 5.8SrDNA phylogeny showing that fungi mycorrhizal with *Dactylorhiza baltica* on oil shale ash hills most likely belong to *Ceratobasidium albasitensis*. Phylogeny constructed with 516 bp and 21 taxa, and midpoint-rooted. The best tree resulting from a heuristic maximum likelihood search in PHYML is presented, with support values derived from 500 bootstrap replicates. Values at nodes indicate percentage bootstrap support.

---

(Otero et al., 2002; McCormick et al., 2004; Otero et al., 2004) and that orchid mycorrhizal interactions may persist as highly specialized in times of environmental stress for the plant (McCormick et al., 2006). Phylogenetically, the fungal species identified in this study run the wide, polyphyletic breadth of orchid mycorrhizal fungi (Roberts, 1999; Selosse et al., 2004) and generally agree with fungal groups noted as mycorrhizal with other members of these respective orchid genera (Rasmussen, 1995). The conditions on mine tailings hills may represent ecologically novel niches, and the lack of other established plants and
fungi suggests primary succession. Plants in novel habitats often colonize more effectively when connected to common mycorrhizal networks, which serve to redistribute nutrients to seedlings and weaker plants (Nara and Hogetsu, 2004). Orchids receive carbon and a variety of other nutrients from their mycorrhizal fungi (Bidartondo et al., 2004; Cameron et al., 2006), and mycorrhizal fungi of *Epipactis atrorubens* are known to accumulate heavy metals on mine tailings sites (Jurkiewicz et al., 2001). Because these orchids often grew within 0.5 m of each other (R. P. Shefferson, personal observation), we suggest the possibility of a common mycorrhizal network among orchids on mine tailings sites. These mycorrhizal fungi may aid in the development of a successional front from which further colonization may take place and onto which perhaps even other plant species may establish (Nara, 2006).

The demonstration that these particular fungi exist in mycorrhizal associations on mine tailings hills advances our knowledge of these little-studied organisms. *Ceratobasidium albatennis* is a recently described fungus occurring in Europe (González et al., 2002) and is known to promote seedling growth of some plant species as a root endophyte (Grönberg et al., 2006). Although many *Ceratobasidium* spp. are plant parasites, many are also orchid mycorrhizal and/or saprotrophic (Roberts, 1999). The unnamed members of the Tulasnellaceae found to be mycorrhizal with *Epipactis atrorubens* and *Orchis militaris* are closely related to known mycorrhizal associates of some Cypripedium species (Shefferson et al., 2005), as well as of some epiphytic orchids from the Andes (Suárez et al., 2006; Shefferson et al., 2007). The ascomycetes mycorrhizal with *E. atrorubens* appear closely related to fungi occasionally mycorrhizal with *E. microphylla* (Sellesse et al., 2004), suggesting that they may be common associates of this orchid genus.

The fungi found to be mycorrhizal associates of these plants include fungi sometimes referred to as dark septate endophytes, or DSEs. DSEs are common mycorrhizal associates of plants at the leading edge of primary succession (Cazares et al., 2005), but they may also be other kinds of endophytes (Clay, 1990; Jumpponen, 2001; Cheplick and Cho, 2003; Bayman and Otero, 2006). *Geopora cooperi* and *Trichophaea woolhopeia*, the two species that may be characterized in this way, are both members of the ascomycetous order Pezizales, with the former forming hypogeous and the latter forming epigeous fruiting bodies. Both are ectomycorrhizal with a variety of northern forest trees, including *Pinus* spp. (Smith et al., 2006; Tedersoo et al., 2006). In addition to these mycorrhizal fungi, we documented the poorly studied ascomycete *Chalara dualis* in one sample of *Epipactis atrorubens*. However, because its closest relatives are generally plant parasites, we assume that its occurrence is not due to mycorrhizal interaction but instead parasitism, as occurs in other orchids as well (Shefferson et al., 2005). Further ecological study will be necessary to flesh out whether these endophytes are truly mycorrhizal.

In this study, we showed that mycorrhizal fungi occur in orchids colonizing the novel and generally desolate ash sites resulting from oil shale mining and identified some of these fungi. However, our inability to sample greater numbers of both plants and localities prevents us from assessing whether mycorrhizal colonization differs in more subtle ways between pristine sites and mine tailings sites. We suggest further study on mycorrhizal orchids in mine sites to determine whether mycorrhizal fungi confer resistance to toxic metals. We also suggest further work on microbial interactions in plants at the forefront of primary succession.

**LITERATURE CITED**


