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A comparison of ectomycorrhiza identification based on morphotyping and PCR-RFLP analysis

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Two methods are currently being used to describe ECM fungal communities associated with root tips: molecular techniques and morphological classification. Previous studies have found that these two approaches give conflicting results, with several fungal genotypes being identified from different ectomycorrhizas within the same morphotype. This has led researchers to question the usefulness of the morphological approach. The objective of this study was to compare the two approaches on ectomycorrhizas collected from three plant species growing in two different environments. Specifically, mycorrhizas were classified using a detailed morphological approach and then were subjected to PCR-RFLP analysis of the ITS region of the rRNA gene repeat. Ectomycorrhizas of Douglas-fir (*Pseudotsuga menziesii*) and paper birch (*Betula papyrifera*) were sampled from three widely dispersed sites with different soil types in the southern interior of British Columbia. Ectomycorrhizas of Douglas-fir and arbutoid mycorrhizas from *Arctostaphylos uva-ursi* were sampled from a fourth site in a different biogeoclimatic zone. For eight of eleven dominant morphotypes, one main RFLP banding pattern was observed. Ninety-three % of the mycorrhizas analyzed in these eight morphotypes would have been classified in the same way by either method. Five of the eight morphotypes were positively identified as *Russula nigricans*, *Lactarius* sp., *Leccinum scabrum*, *Rhizopogon* sect. *Villosuli*, and *Thelephora terrestris* by matching the RFLPs to those of fungal fruit bodies in our database or by sequencing the ITS region. The other morphotypes producing one dominant RFLP pattern were designated as *Cenococcum*, E-strain and *Mycelium radicis atrovirens* (MRA) based on their morphology. Morphotyping did not distinguish amongst major RFLP types for mycorrhizas classified as *Amphinema*-like, *Piloderma*-like and *Rhizopogon*-like A. We conclude that detailed morphological classification can be very useful as the primary method of ectomycorrhizal classification, when used in conjunction with molecular techniques. This approach will allow for an efficient use of research funds.

INTRODUCTION

Different methods can be used to determine the diversity and structure of ectomycorrhizal (ECM) fungal communities. Traditionally, surveys of above-ground fruit bodies have been the most common method because the sporocarps could be identified to species using standard taxonomic approaches. With time, it has become clear that these surveys do not accurately reflect the diversity or species composition of below-ground ECM fungal communities (e.g. Mehmann *et al.* 1995, Gardes & Bruns 1996, Dahlberg *et al.* 1997, Jonsson *et al.* 1999a, van der Heijden 1999, Zhou *et al.* 2001). This may be because some ECM fungi, especially members of the *Ascomycota*, *Corticiaceae*, and *Thelephoraceae* produce small or cryptic fruit bodies that are overlooked during fruit body surveys. Other ECM fungi may be missed from such surveys because they produce hypogeous fruit bodies or have no known sexual state (e.g. *Cenococcum geophilum*). Further, the production of fruit bodies is dependent upon environmental conditions such as moisture and temperature, and thus fungi do not produce fruit bodies every year. Therefore, when describing ECM fungal communities, it is clear that the ectomycorrhizas themselves must be studied in order to determine the fungi that are actively associated with plant roots.

Two methods are currently being used to describe ECM fungal communities associated with root tips: molecular techniques and morphological classification. The molecular techniques are based primarily on polymerase chain reaction (PCR) amplification of regions of fungal ribosomal DNA (rDNA). Morphological classification involves examination of the

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entire mycorrhiza under stereo and/or compound microscopes. In most studies, both techniques are used to some extent, but with one predominating. For example, ectomycorrhizas are usually separated into groups prior to molecular analysis by examining a small number of characters under a stereomicroscope (e.g. Horton & Bruns 2001). Researchers who use detailed morphotyping as their primary means of classification often rely on molecular techniques to confirm the classification of the fungi (e.g. Hagerman *et al*. 1999b). While both techniques have advantages and disadvantages, it has been suggested that morphological classification is not precise enough to accurately describe ECM fungal communities (Nylund *et al*. 1995, Mehmann *et al*. 1995), is too time consuming to learn (Dahlberg 2001), and may not be consistent amongst laboratories. These concerns arise, in part, from poor correlations between the two methods (Nylund *et al*. 1995, Kårén *et al*. 1997, Jonsson *et al*. 1999a, b, Erland *et al*. 1999, Mah *et al*. 2001).

Molecular techniques are based on the observation that the internal transcribed spacer (ITS) region of nuclear rDNA exhibits a high level of variability amongst ECM fungal species and minimal variation within species, and thus is a good candidate for distinguishing ectomycorrhizal taxa (Egger 1995, Gardes & Bruns 1996). When fungal DNA is amplified from an ectomycorrhiza, restriction fragment length polymorphisms (RFLPs) generated by restriction enzyme digests of the ITS region can be used to separate most, but not all, ECM fungal species (Gardes & Bruns 1996, Kårén & Nylund 1997, Farmer & Sylvia 1998, Horton & Bruns 1998, Jonsson *et al*. 1999a, Aanen, Kuyper & Hoekstra 2001). Where fruit bodies are available, RFLPs from these can be compared with those from mycorrhizas to identify the fungal symbiont. If mycorrhizal RFLPs do not match fruit body RFLPs, the ML5/ML6 region of the mitochondrial large subunit (mt Lsu) rDNA can be amplified, sequenced, and compared with a published database (Bruns *et al*. 1998, Horton & Bruns 2001) to place the fungus in a family. In addition, sequencing of the ITS region has been used to determine the taxonomic affinity of ECM fungi (Chambers, Sawyer & Cairney 1999). Thus, the two major advantages of molecular analyses are: (1) that it may be possible to identify the fungus with reasonable certainty (Goodman, Durall & Trofymow 1996); and (2) that both inter- and intraspecific genetic variability can be evaluated (Dahlberg 2001). A disadvantage is that the success rate of DNA extraction and amplification can vary amongst fungi. For this reason, the use of molecular techniques on randomly sampled root tips, with no prior morphological categorization, cannot be used to generate quantitative descriptions of ECM fungal communities. Furthermore, because of the cost and time involved, it is practical to amplify DNA from only a small subset of ectomycorrhizas sampled.

Morphotyping has been used for many years to distinguish ectomycorrhizas formed by different fungi (Zak 1973, Haug & Oberwinkler 1987). In order for morphotyping to be effective, it is recommended that a considerable number of characters be precisely described under both low and high magnifications (Goodman *et al*. 1996). From the 1980s onward, several research groups have collected and published standard descriptions of ectomycorrhizas from Europe and North America (Agerer *et al*. 1987–98, Ingleby *et al*. 1990, Goodman *et al*. 1996–2000), facilitating the use of this technique. Morphotyping of ectomycorrhizas has been used successfully to study host specificity (Simard *et al*. 1997, Bradbury 1998, Massicotte *et al*. 1999), ectomycorrhizal physiology (Wallender *et al*. 1997, Wallenda & Read 1999, Conn & Dighton 2000), succession (Danielson 1991, Visser 1995), the effects of silviculture practices (Jones *et al*. 1997, Kranabetter & Wylie 1998, Hagerman *et al*. 1999b), fertilization (Fransson, Taylor & Finlay 2000), elevated carbon dioxide (Rygiewicz, Martin & Tuininga 2000), and pollution (Roth & Fahey 1998) on ECM fungal communities. Morphotyping has also been an effective method for selecting closely-related ECM fungi for further genetic study (Vrålstad, Fossheim & Schumacher 2000). This approach has the advantage that many root tips can be examined in a relatively short period of time (hundreds per day). Given their heterogeneous distribution in soil (Brundrett & Abbott 1995, Stendell, Horton & Bruns 1999, Bidartondo *et al*. 2000, Horton & Bruns 2001), it is essential to study a large number of ectomycorrhizas in order to thoroughly understand ectomycorrhizal fungal communities. Definitive identification of the fungus forming the morphotype can be done either by tracing mycelia from the root tip to a fruit body (Agerer 1987–98), something that is difficult to do, or by the molecular techniques described above. The accuracy and speed of morphotyping depends greatly on the skill of the individual performing the analysis and experience is required to gain this type of expertise. Molecular techniques can be very useful in confirming the accuracy of classification during this training period (Horton & Bruns 1998). Nevertheless, some fungi that can be distinguished from each other by PCR-RFLP cannot be separated morphologically, even after very careful examination (Eberhardt Walter & Kottke 1999). It should be noted that the same is true of fruit bodies, perhaps because genetic differences appear prior to morphological differentiation in fungi (Taylor *et al*. 2000). Furthermore, relatively little is known about levels of phenotypic variation of ectomycorrhizas from different hosts and under different environmental conditions (Egger 1995).

The objective of this study was to test the accuracy of ECM morphological groupings by using PCR-RFLP methods on ectomycorrhizas collected from different plant hosts and under various habitats. Earlier studies that grouped ectomycorrhizas by macroscopic observation only (Erland *et al*. 1999, Jonsson *et al*.

1999a, b) or by more detailed examination (Mah *et al.* 2001) found that some morphotypes produced more than one RFLP pattern and that the same RFLP pattern could be found in more than one morphotype. Others have found that detailed morphotyping can be used to consistently distinguish ectomycorrhizas formed by congeneric *Lactarius* or *Russula* species (Kernaghan, Currah & Bayer 1997, Pritsch *et al.* 1997). Some researchers find that even crude morphotyping can effectively separate mycorrhizae formed by different fungal genotypes, at least within individual soil samples (Horton & Bruns 2001). We sampled ectomycorrhizas of Douglas-fir (*Pseudotsuga menziesii*) and paper birch (*Betula papyrifera*) at three widely dispersed sites in the Interior Cedar-Hemlock (ICH) biogeoclimatic zone of southern British Columbia, Canada, as well as ectomycorrhizas of Douglas-fir and arbutoid mycorrhizas of *Arctostaphylos uva-ursi* from the Interior Douglas-fir (IDF) zone, also in the southern interior of British Columbia.

MATERIALS AND METHODS

Study sites

Mixture sites

Ectomycorrhizal root tips were sampled in late Sept. 1997, from six-year-old Douglas-fir and paper birch trees that had been planted as part of a long-term study investigating regeneration of mixed stands; see Jones *et al.* (1997) for work on earlier mycorrhiza samples from these sites and for a more complete description of the study. The study comprises three replicate sites ranging in elevation from 650 to 750 m in the ICH zone of the southern interior of British Columbia, with the two tree species planted in a range of proportions and densities. Two sites, Adams Lake (51° 28′ N, 119° 30′ W) and Malakwa (50° 58′ N, 118° 44′ W), are located in the Thompson Moist Warm ICH (ICHmw3) variant (Lloyd *et al.* 1990) and have Humo-Ferric Podzol soils (Soil Classification Working Group 1998). The Hidden Lake site (50° 34′ N, 118° 50′ W) is located in the Shuswap Moist Warm ICH (ICHmw2) variant and has a Dystric Brunisol soil. The sites were clear-cut in 1978 (Hidden Lake), 1987 (Adams Lake), and 1988 (Malakwa). Roots of Douglas-fir and paper birch were collected from 12 randomly selected seedlings of Douglas-fir and 12 of paper birch from each of five 40×40 m plots at each site, for a total of 180 seedlings of each species. We loosened the soil at the base of the trees and then sampled only those lateral roots that could be traced to the main stem.

Opax Mountain sites

Mycorrhizas were sampled from the Opax Mountain Silvicultural Systems Trial (50° 35′ N, 120° 74′ W) as part of a larger study investigating plant species that can act as refugia for ECM fungi during stand regeneration (see Hagerman, Sakakibara & Durall 2001 for further results). The study area consisted of two replicate sites ranging in elevation from 950–1370 m (Bealle-Statland 1998). The upper elevation site (1200–1370 m) is in the Dry Cool IDF (IDFdk1) variant (Lolyd *et al.* 1990) and has Brunisolic Gray Luvisol soils (Soil Classification Working Group 1998). The lower elevation site (950–1100 m) is in the Very Dry Hot IDF (IDF × h2) variant and soils were classified as Orthic Gray Luvisols and Orthic Eutric Brunisols. Five individual plants each of Douglas-fir and *A. uva-ursi* were randomly sampled in three 1.6 ha patch cuts (logged in the winter of 1993–94) in each of the upper elevation and lower elevation replicate sites and in adjacent uncut forest in the fall of 1997 and 1998 ($n = 120$ in each year).

Morphological typing of mycorrhizas

Roots were stored 5 °C until examined, with a maximum storage period of five months. At that time, the roots were washed over a 2 mm sieve and the fine laterals cut into lengths of approximately 1 cm. Root segments were spread in water in a glass dish and two hundred live mycorrhizas were removed from randomly selected root segments and examined individually in order to categorize them as a particular mycorrhizal morphotype.

Characteristics used to categorize the tips into consistently recognizable morphological types were those described by Goodman *et al.* (1996). Colour and texture of mycorrhizal tips, colour and abundance of external hyphae, and the presence and colour of hyphal strands were described while examining root tips under a stereomicroscope at 40 ×. Representative mycorrhizas were also observed under 400 × or 1000 × magnification either as whole mounts (entire root tip) or as mantle peels (only the fungus). Mantle peels were made by separating the fungal tissue from the root with fine forceps. The appearance of the inner and outer mantle surface (felt or net prosenchyma; net, irregular interlocking, irregular non-interlocking, or regular synenchyma); the size of hyphal elements in the mantle; the diameter, surface texture, and frequency of clamps on external hyphae; and the presence and type of cystidia were described. Our descriptions were compared with other published descriptions of ectomycorrhizas (e.g. Agerer 1987–98, Ingleby *et al.* 1990, Goodman *et al.* 1996–2000) for tentative initial identification of the fungal symbionts. Photographs and frozen specimens from both study sites are archived at Okanagan University College, Kelowna, British Columbia.

Sampling for DNA analysis

The seven most common ectomycorrhizal morphotypes from the Mixture sites (*Cenococcum*-like, E-strain, *Leccinum*, *Mycelium radicis atrovirens* (MRA), *Rhizopogon A*, *Rhizopogon B* and *Thelephora*) were selected

for DNA analysis. Sampling was performed on a subset of the root-tips that were examined for morphotyping. From amongst all seedlings sampled, five seedlings per species per site were randomly selected, and 200 live mycorrhizas per seedling were separated into morphotypes. As the classification was being done, ectomycorrhizas from each morphotype were further subdivided into three subsets that represented the range of morphological variability within a morphotype. Then tips from the five seedlings per site were combined and root tips were randomly selected from each subgroup of each morphotype for DNA analysis.

Samples from 15 different morphotypes from Douglas-fir or *Arctostaphylos uva-ursi* at the Opax sites underwent PCR-RFLP analysis (see Hagerman *et al.* 2001). Representative root tips of each morphotype were selected for DNA extraction. Here we present the results from the eight most common morphotypes at Opax (*Amphinema*-like, *Cenococcum*-like, E-strain, *Lactarius*, *Piloderma*-like, *Rhizopogon*-like A, *Russula*, and *Rhizopogon*-like B). Although *A. uva-ursi* forms arbutoid mycorrhizas, the mantles were as well developed as the mantles of Douglas-fir.

Four morphotypes (*Cenococcum*-like, E-strain, *Rhizopogon*-like A, and *Rhizopogon*-like B) were common between the Mixture and Opax sites; thus, 11 morphotypes were studied in total, between the two studies. Descriptions of the *Amphinema*-like, *Lactarius* (OUC 143), *Piloderma*-like, *Rhizopogon* and *Russula nigricans* morphotypes have been published in Hagerman *et al.* (2001); *Thelephora* and MRA morphotypes are described in Jones *et al.* (1997); and the *Cenococcum*-like and E-strain morphotypes are described in both the above papers. Note that two E-strain morphotypes, OUC 060 and OUC 061, are described in Jones *et al.* (1997). PCR-RFLP analysis done subsequent to the publication of this paper showed that these two morphotypes were formed by the same fungal genotype and this is presented as OUC 060 in the present study. *Leccinum scabrum* mycorrhizas are illustrated in Agerer (1987–98).

DNA isolation, PCR amplification and restriction enzyme digest

Total genomic DNA was isolated from air-dried frozen (−20 °) root tips as reported previously (Baldwin & Egger 1996, Hagerman *et al.* 1999b). Following extraction, the internal transcribed spacer (ITS) region of the fungal nuclear rDNA was specifically amplified by the primers ITS1 (5′TCCGTAGGTGAACCTGCGG3′) (White *et al.* 1990) and NL6bmun (5′CAAGCGTTTCCCTTTCAACA3′) (Egger 1995). A typical PCR amplification reaction consisted of the following components; 4 μl template DNA, 17.2 μl sterile distilled water, 0.188 mM deoxyribonucleotides, 3 μl 10 × PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl), 1.875 mM $MgCl_2$, 0.375 μM each primer, and 0.8 U Expand High Fidelity PCR System (Boeringher Mannheim). In general, DNA preparations were used in PCR reactions either undiluted or diluted by one-half. Our experience has shown that, with this combination of primers, further dilution does not result in increased amplification success. Samples were amplified using a Perkin Elmer DNA Thermal Cycler. A seven minute hot start was followed by PCR cycling as follows: one minute 95 ° followed by 35 cycles of denaturation at 94 ° for 45 s, annealing at 48 ° for 45 seconds, ramping to 72 ° for 55 s with a one second extension after each cycle, and extension at 72 ° for 2 min and 10 s. A final extension step was added for seven min at 72 °, and then the temperature was held at 4 °. PCR products were visualized on agarose gels, 1.5% in concentration.

Three restriction enzymes, *Alu* I, *Hin*f I, and *Mbo* I, were used. Digests were performed in a total volume of 20 μl, consisting of 17 μl of PCR product, 2 μl of REact buffer, and 10U of enzyme, then resolved on a 2.5% aggregate gel consisting of 1% agarose and 1.5% NuSieve (FMC Bio Products) by electrophoresing at 70 V for 5 h. Gels were stained for 45 min in ethidium bromide, destained in distilled water for 20 min, and photographed on an UV-transilluminator. RFLP band sizes were estimated by comparison to a standard 100 base pair (bp) molecular weight ladder. Samples of the same morphotype that were run on different gels were recorded as generating identical RFLP patterns when no variation was discernible upon manual comparison with the 100 bp molecular standard. Banding patterns were compared to RFLPs previously generated from sporocarps collected in the ICH zone of British Columbia (unpubl.) and from ectomycorrhizal root tips collected in other studies (Jones *et al.* 1997, Durall *et al.* 1999, Hagerman *et al.* 1999a, b).

DNA sequencing

Partial ITS sequences were determined for two samples of *Rhizopogon* sp. A – RFLP pattern I, two samples of *Rhizopogon* sp. A – RFLP pattern II, one sample of *Rhizopogon* sp. A – RFLP pattern III and one of *Rhizopogon* B – RFLP pattern I. The DNA extraction and PCR procedures were as described above (Baldwin & Egger 1996, Hagerman *et al.* 1999b), except that the primers ITS1-F (5′CTTGGTCATTTAGAGGAAGTAA3′) and ITS4-B (5′CAGGAGACTTGTACACGGTCCAG3′) were used instead (Gardes & Bruns 1993). PCR products were quantified and sent to the University of British Columbia Nucleic Acid and Protein Services Unit for automated sequencing using the primer ITS1-F.

RESULTS

We generated RFLPs from 10 to 23 mycorrhizas of each morphotype in each study (Table 1). For eight of the 11 morphotypes examined, one banding pattern predominated. For the *Lactarius* and *Russula* morpho-

Table 1. Summary of ectomycorrhizas analyzed by PCR-RFLP.

OUC morphotype no.	Mycorrhizal morphotype	No. of tips generating RFLPs	Success rate (%)	No. of tips from Douglas-fir host	No. of tips from second host	No. of different RFLP patterns (number of tips per pattern)
Mixture						
30	*Cenococcum*-like	20	38	14	6	2 (19+1*)
60	*E-strain*	19	58	15	4	1 (19)
160	*Leccinum*	12	35	n.p.	12	2 (10+2*)
170	MRA	10	21	7	3	2 (8+2)
210	*Rhizopogon* A	18	75	18	n.p.	4 (9+7+1*+1)
230	*Rhizopogon* B	14	27	14	n.p.	3 (11+2*+1)
240a	*Thelephora*	22	67	11	11	2 (20+2*)
	Total	115		79	36	
Opax						
20	*Amphinema*-like	11	61	7	4	2 (7+4)
30	*Cenococcum*-like	11	61	7	4	1 (11)
60	*E-strain*	17	74	10	7	2 (15+2)
143	*Lactarius*	15	71	15	n.p.	1 (15)
200	*Piloderma*-like	23	75	12	11	5 (7+5+4+4+3)
210	*Rhizopogon* A	10	63	10	n.p.	4 (2+1+4+3*)
220a	*Russula*	11	58	8	3	1 (11)
230	*Rhizopogon* B	14	56	14	n.p.	1 (14)
	Total	112		83	29	

[1] *Betula papyrifera* for the Mixture study; *Arctostaphylos uva-ursi* for the Opax study.
* This RFLP banding pattern was found on all morphotypes marked with an asterisk (*).
n.p. – no mycorrhizas of this type were present on this host.

types, every root tip sampled from within a morphotype gave rise to the same RFLP. For tips classified as *Cenococcum*-like, E-strain, *Leccinum*, MRA, *Rhizopogon* B or *Thelephora*, a clear majority (at least 80%) of the tips within each morphotype produced the same RFLP (Table 2). Thus, these eight morphotypes produced consistent patterns in 153 of the 165 root tips (93%) subjected to RFLP analysis. This consistency in RFLP patterns occurred across different host species, from both ectomycorrhizas and arbutoid mycorrhizas, and across the four study sites, in cases where morphotypes were common to both studies. This occurred even though we selected from across the range of morphological variation within the morphotypes from the Mixture study.

Much of the within-morphotype variation in RFLPs could be attributed to a single, aberrant RFLP pattern that was generated from several root tips in most morphotypes from the Mixture sites (* in Tables 1–2). In the cases of the *Cenococcum*-like, *Leccinum*- and *Thelephora* morphotypes, this pattern accounted for all of the within-morphotype variation that was observed. This banding pattern was also seen in some tips classified as the two *Rhizopogon*-like types. The fungus producing this pattern is not known. It should be noted that if this aberrant RFLP pattern was from a common additional fungus associated with the mycorrhizas, it does not necessarily indicate that these mycorrhizas were misclassified. Therefore, the consistency between morphotyping and molecular typing could have been as high as 160 of 165 root tips, or 97% for these eight morphotypes.

Morphotyping did not distinguish amongst major RFLP types for mycorrhizas classified as *Amphinema*-like, *Piloderma*-like and *Rhizopogon* A. In the case of the *Piloderma*-like morphotype, five fairly similar RFLP patterns were produced, each differing from the others in the digests of one or two enzymes (Table 2). For *Rhizopogon* A, three of the five RFLP patterns, labelled as I, II, and III in Table 2, accounted for 91% of the variation seen in this group. Patterns II and III were identical except for the presence of an additional 475 bp band in the *Alu* I digest of Type II tips. This band was often observed to fluoresce less strongly than other bands of similar size in the digest.

To further explore these findings, a subsample of tips giving rise to *Rhizopogon* A patterns I, II and III and *Rhizopogon* B pattern I were subjected to DNA sequence analysis in the ITS region. Using the primer ITS1-F, 650 to 700 bases of sequence were amplified from the ITS region for each sample. Sequences generated from ectomycorrhizal root tips were compared with ITS sequences generated from fruiting bodies of known *Rhizopogon* species. The sequence from *Rhizopogon* A pattern I was found to differ from that of *R. gilkeyae* in only one base (L. Grubisha, pers. comm.). The sequence from *Rhizopogon* A pattern II nested just outside that of the *R. vinicolor* group and the sequence of *Rhizopogon* A pattern III nested with that of the *R. vinicolor* group. Thus, it appears that the *Rhizopogon*-like A morphotype was formed by two or more *Rhizopogon* genotypes in sect. *Fulviglebae*. *Rhizopogon* B pattern I also grouped with the genus *Rhizopogon*, in sect. *Villosuli*.

Four morphotypes were positively identified to

Table 2. Approximate restriction fragment sizes (bp) of the ITS region of ectomycorrhizal morphotypes.

OUC morphotype description no.	Study[1]	Mycorrhizal morphotype	No. of tips per RFLP pattern	Approximate band sizes (bp)												
				Undigested *Alu* I						*Hinf* I				*Mbo* I		
20	O	*Amphinema*-like (I)[4]	8	1000	575	195				335	285	165	150	800	200	
20	O	*Amphinema*-like (II)	4	1000	575	195				285	250	165	150	800	200	
30	M/O	*Cenococcum*-like (I)	30	900	435	150	150	125		275	165	135	**90**[23]	305	225	150
30*	M	*Cenococcum*-like (II)	1	900	550	125	115			435	295	165		300	215	200
60	M	E-strain (I)	36	950	395	265	185	115		500	185	175	135	325	**225**	150
60	O	E-strain (II)	2	1025	425	185	125			375	325	165		450	375	225
143	O	*Lactarius* sp.[2,5]	14	1125	515	285	195			415	350	165	150	575	280	150
160	M	*Leccinum scabrum* (I)[2]	10	~ 1800	525	365	190	140	100	950	435	165	110	850	500	225
160*	M	*Leccinum*-like (II)	2	900	550	125	115			435	295	165		300	215	200
170	M	MRA (I)	8	900	625	150	125			450	260	160		365	315	190
170	M	MRA (II)	2	900	385	250	150	125		450	260	160		365	315	190
200	O	*Piloderma*-like (I)	5	1000	625	195	125			315	175	165		350	225	195
200	O	*Piloderma*-like (II)	4	1000	625	195	125			315	175	165		500	225	175
200	O	*Piloderma*-like (III)	4	1025	375	275	195	125		315	175	165		500	225	175
200	O	*P. byssinum* (IV)[2]	7	1025	375	275	195	125		315	175	165		500	300	225
200	O	*Piloderma*-like (V)	3	1025	375	275	195	125		315	250	175	165	815	225	
210	M/O	*Rhizopogon* A (I) Section Fulviglebae	11	1125	765	185	115			205	165	**135**	110	315	**250**	240
210	M/O	*Rhizopogon* A (II) Section Fulviglebae	8	1125	475	425	285	185	115	205	165	**135**	110	550	250	225
210	O	*Rhizopogon* A (III) Section Fulviglebae	4	1125	425	285	185	115		205	165	**135**	110	550	250	225
210*	M/O	*Rhizopogon*-like A (IV)	4	900	550	125	115			435	295	165		300	215	200
210	M	*Rhizopogon*-like A (V)	1	985	375	175	165	125	90	365	200	**170**		585	195	
220a	O	*Russula nigricans*[2]	11	1050	475	185	140	125		375	325	165	150	565	200	165
230	M/O	*Rhizopogon* B (I) Section Villosuli	25	1075	715	185	125			225	165	**130**	105	550	250	225
230*	M	*Rhizopogon*-like B (II)	2	900	550	125	115			435	295	165		300	215	200
230	M	*Rhizopogon*-like B (III)	1	925	365	235	150			435	275	165		365	315	175
240a	M	*Thelephora terrestris* (I)[2]	20	1050	575	185	165	125		325	265	**170**	105	450	375	235
240a*	M	*Thelephora*-like (II)	2	900	550	125	115			435	295	165		300	215	200

* This RFLP banding pattern was found on all morphotypes marked with an asterisk (*).

[1] M, Mixture study; O, Opax Study.

[2] These RFLPs matched those of sporocarps of the fungus listed.

[3] Numbers in boldface indicate the probable presence of two bands of this size.

[4] Roman numerals represent different RFLPs for the same morphotype.

[5] Fruit bodies in our database identified as *Lactarius rubrilacteus*, *L. vietus*, *L. alnicola*, *L. scrobiculatus*, *L. glyciosmus*, *L. pseudomucidus* all produced the same RFLP, and this matched the one generated by the *Lactarius* tips sampled here.

Russula nigricans, *Leccinum scabrum*, *Piloderma byssinum* or *Thelephora terrestris* by matching the RFLPs to those of fungal fruit bodies in our database (Table 2). The RFLP generated by the *Lactarius* morphotype matched a single pattern generated by basidiomer identified as six different species of *Lactarius* (Table 2). These six species were collected from the same biogeoclimatic zone as the Mixture sites. The main MRA RFLP pattern did not match with those generated from fruit bodies of *Phialocephala fortinii* (K. Williams, pers. comm.). Further work is currently underway to identify this ascomycete.

DISCUSSION

The major conclusion from this study is that morphotyping can be used to accurately group ectomycorrhizas formed by the same fungal species for many, but not all fungi. We found that at least 93% of the mycorrhizas from eight of the 11 morphotypes were classified in a way consistent with PCR-RFLP analysis. Nevertheless, this study and others (Pritsch *et al*. 1997, Eberhardt *et al*. 1999, Mah *et al*. 2001) show that, even if morphotyping is done at a very detailed level, it will not be able to distinguish amongst ectomycorrhizas formed by some species of fungi. More extensive use of PCR-RFLP is required to quantify the abundances of these ectomycorrhizas. Of course PCR-RFLP analysis of the ITS region cannot always distinguish amongst fungal species either, as found by us for the species of *Lactarius* listed in Table 2 and by Kårén *et al*. (1997) for numerous species of *Cortinarius*. Sequencing of the ITS region will likely help with problem genera such as *Cortinarius* and *Lactarius* (Horton & Bruns 2001).

Our conclusion that accurate morphotyping can produce results that are in agreement with molecular results are consistent with those of Pritsch *et al*. (1997). These authors used PCR-RFLP analysis to identify 15 morphotypes, previously described in detail, from *Alnus glutinosa*. Using five restriction enzymes and two to seven tips per morphotype, they found that 14 of the morphotypes could not be separated further with PCR-RFLP of the ITS region. Only mycorrhizas formed by *Naucoria escharoides* and *N. subconspersa* could not be distinguished from each other using morphology. With experience, Horton & Bruns (1998) also found excellent agreement between morphotypes and RFLP types, even though morphological classification was performed under the stereomicroscope. Horton & Bruns (2001) suggest that this crude type of morphotyping works very well for separation of genotypes within a soil sample, especially when confirmed with molecular techniques, but is not sufficient for separation across samples or sites.

Our results differ from those of Mah *et al*. (2001) who conducted detailed morphological analysis combined with PCR-RFLP analysis of the ITS region for a relatively large proportion (10%) of the mycorrhizas examined. Those authors found poor agreement between morphological and molecular classification. Only one of eight major morphotypes, *Cenococcum*, produced the same RFLP from all root tips. The remaining seven morphotypes comprised from two to six RFLP types. Mah *et al*. (2001) may have found more genotypes within each morphotype than we did because they extracted DNA from a higher proportion of sampled root tips; however, detailed examination of the results suggests that this is not the explanation. In our study, eight of the 11 morphotypes had one predominant set of RFLP patterns, representing from 80 to 100% of root tips (ignoring the aberrant RFLP pattern indicated with an asterisk in Table 2). In the Mah *et al*. (2001) study, the largest proportion of tips that produced the dominant RFLP pattern for a morphotype was 67% (except for *Cenococcum*) and the less common patterns still represented sizeable proportions of the sample, usually 20–35% for each minor pattern. Because the minor patterns were generated from such a high proportion of root tips, we would have detected these, even with our smaller sample sizes. Moreover, even though our sample sizes were lower, we deliberately sampled from across obvious sources of variability such as host species, latitude, climatic regime, soil type, mycorrhiza type (ectomycorrhizas vs. arbutoid mycorrhizas) and range of morphological variation (for the mixture study). Our finding that the major RFLPs generated by *Amphinema*-like, *Cenococcum*-like, MRA, and *Thelephora* mycorrhizas, plus RFLP IV of *Piloderma* mycorrhizas matched those of the same morphotypes on *Picea engelmanii* × *glauca* growing in high elevation forests (Hagerman *et al*. 1999b) is further evidence that morphotyping can consistently group ectomycorrhizas formed by the same fungus, but on different hosts. The fungus forming the E-strain mycorrhizas differed, however, between the higher elevation community (Hagerman *et al*. 1999b) and the communities sampled here.

Consistency in morphotyping between laboratories is illustrated by the finding that the RFLPs generated by five of the morphotypes in the present study appear to match RFLPs for the same morphotype as classified by Mah *et al*. (2001). For *Alu* I and *Hin*f I, the restriction enzymes in common between the two studies, the following RFLPs were similar, where the first pattern listed is from our study and the second pattern is from Mah *et al*. (2001): *Amphinema* I, *Amphinema* genotype 1c; *Cenococcum* I, *Cenococcum* genotype 1; MRA I, MRA genotypes 1b and 2; MRA II, MRA genotypes 3 and 4; *Piloderma* III and IV, *Piloderma* genotype 2; *Thelephora* I, and *Thelephora* genotype 2. Therefore, we feel that our conclusions are robust, although there is no question that more genotypes with a relative abundances less than 10% would be detected using the extensive analyses carried out by Mah *et al*. (2001). Our approach will adequately sample the dominant ectomycorrhizal fungi in a community, which, according to Horton & Bruns (2001), is the most reasonable goal with current technology.

A second possible explanation for differences between our study and Mah *et al*. (2001) is that we missed some minor patterns because our gels were scored manually rather than with the aid of a computer program. This explanation can also be discounted because the differences between RFLP patterns observed by Mah *et al*. (2001) for the same morphotype were usually very great (extra bands or bands differing in size by 50%). A third possible explanation for differences between the two studies is that the morphotyping was done to a different degree of precision. Most of the disagreement between molecular and morphological approaches in earlier studies is probably because morphotyping was done only at a low magnification, but Mah *et al*. (2001) used the same detailed set of characters as employed in our study. In conclusion, it is not clear why the results from these two studies, performed in the same part of the world and using similar approaches, differed to such an extent.

Given the usefulness of the morphological approach, we suggest that a more detailed approach to morphotyping would add accuracy to many studies where PCR-RFLP is used as the primary method of describing the ECM fungi community. During these studies, mycorrhizas are typically sorted morphologically under a stereomicroscope using a limited number of macroscopic characters such as colour and texture of fungal mantle, and presence and appearance of extramatrical hyphae and rhizomorphs, and then DNA is amplified from a small subset, typically 2 to 10% of each morphotype (e.g. Jonsson *et al*. 1999a, b). Accurate morphological classification of ectomycorrhizas is especially important in these cases because the morphotypes are used as the basis for scaling up the results of the DNA analysis from the extracted subset to the total number of root tips examined (Horton & Bruns 1998, Jonsson *et al*. 1999a, b). Yet PCR-RFLP groupings and those produced by morphotyping often correlate poorly using these methods. This is not surprising given that a detailed examination of representative tips under at least 400× magnification is required in order to see many of the characters required to accurately classify morphotypes (Agerer 1987–98, Goodman *et al*. 1996). Interestingly, Horton & Bruns (1998, 2001) found very good agreement between crude morphotyping and molecular groupings within the same soil sample.

Arguments are made that morphotyping is very time-consuming and therefore, expensive. There is no question that accurate morphotyping is a skill that takes time to develop (Dahlberg *et al*. 2001), but our experience is that an average of 400 ectomycorrhizas per day can be classified. This includes the time to train a new person, to take the detailed measurements necessary for an accurate description, and to photograph representative tips. After experience has been gained with a set of samples, only a small subsample of tips must be examined at a higher magnification. The advantage of this approach is that large numbers of root tips can be examined, something that is essential in natural systems where the distribution of ectomycorrhizae is very heterogeneous.

In our study, morphotypes designated as *Amphinema*-like, *Rhizopogon*- A, and *Piloderma*-like clearly encompassed several well-represented PCR-RFLP types, but most of the banding patterns within a morphotype appeared to be related. The differences observed could be attributed to: (1) an insertion or deletion, causing a change in the overall size of the ITS region; and/or (2) a point mutation, which either creates or destroys a recognition site for one of the restriction enzymes, and changes the number of bands observed. These related banding patterns may have been a result of intraspecific variation or may be from two or more related species. Our sequencing of DNA amplified from the *Rhizopogon* morphotypes supports the latter conclusion, but intraspecific variation in the ITS region has been reported by several groups studying ectomycorrhizal fungi (Gardes *et al*. 1991, Henrion, Le Tacon & Martin 1992, Kårén *et al*. 1997, Pritsch *et al*. 1997). The presence or absence of introns in otherwise identical ITS sequences is one source of this variation. Therefore, multiple RFLP patterns from one morphotype should not automatically be interpreted to mean that the mycorrhizas were not formed by the same species (Vrålstad *et al*. 2000). The MRA and *Amphinema*-like morphotypes each gave rise to one main banding pattern and an additional, less frequently observed pattern, which differed from the main one in only one enzyme digest. Within-morphotype variation in the MRA morphotype seemed to be due to a point mutation. The finding that only one species of *Amphinema* (*A. byssoides*) is known in British Columbia (Ginns & Lefebvre 1993) supports the hypothesis that RFLP variation in this morphotype is due to intraspecific variation. Some other researchers have continued to treat ectomycorrhizas differing by only one restriction site together as one group for the purpose of community studies (Horton & Bruns 1998, Baar *et al*. 1999), since the ecological implications of such small genetic differences in the ITS region are unknown (see Read 2000).

The same rare, aberrant banding pattern was observed in five of the 11 morphotypes. The finding that the same unusual banding pattern was observed in several morphotypes of divergent physical characters, and only in small numbers, supports the hypothesis that the DNA samples were contaminated with another type of fungus: a saprotroph or another symbiont that was present on the root tip and was preferentially amplified. This is especially likely given that we diluted the DNA by a maximum of two-fold prior to amplification by PCR. Some ectomycorrhizal fungi are known to co-occur in the mantle. For example, Olsson *et al*. (2000) extracted DNA of *Gomphidius roseus* from the majority of tuberculate mycorrhizas formed by *Suillus bovinus*. The situation here is different, however, because the unknown fungus occurred with several different ectomycorrhizal fungi.

In conclusion, our results show that detailed mor-

phological classification can be very useful as the primary method of mycorrhizal classification, when used in conjunction with molecular techniques. While morphotyping, two types of morphotypes will be encountered: (1) morphotypes within which all ectomycorrhizas comprise the same fungal species; and (2) morphotypes within which different mycorrhizas are formed by two or more fungal species. Molecular techniques are required to distinguish these two types of morphotypes. Once this has been done, research funds can be used more efficiently by doing fewer DNA extractions on morphotypes that produce consistent RFLPs and more on those ectomycorrhizas that cannot be categorized by morphological means alone. Detailed morphological examination (e.g. Goodman *et al.* 1996–2001, Agerer 1987–98), rather than crude separations, makes it more likely that a morphotype will comprise ectomycorrhizas formed by only one fungal species. Therefore, we recommend a more detailed approach even for studies that use molecular techniques as the primary method of classification, but that extract DNA from a small subset of the ectomycorrhizas examined. In most cases, even when morphotyping is very consistent, PCR-RFLP of the ITS region, together with sequencing, will be required to identify the fungal symbiont.

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REFERENCES

Aanen, D. K., Kuyper, T. W. & Hoekstra, R. F. (2001) A widely distributed ITS polymorphism within a biological species of the ectomycorrhizal fungus *Hebeloma velutipes*. *Mycological Research* **105**: 284–290.

Agerer, R. (1987–98) *Colour Atlas of Ectomycorrhizae*. Verlag Eduard Dietenberger. Schwäbisch Gmünd, Einhorn.

Baar, J., Horton, T. R., Kretzer, A. M. & Bruns, T. D. (1999) Mycorrhizal colonization of *Pinus muricata* from resistant propagules after a stand-replacing wildfire. *New Phytologist* **143**: 409–418.

Baldwin, Q. F. & Egger, K. N. (1996) Protocols for analysis of DNA from mycorrhizal roots. In *Concise Descriptions of North American Ectomycorrhizae* (D. M. Goodman, D. M. Durall, J. A. Trofymow & S. M. Berch, eds): 3C.1–3C.2. Mycologue Publications, Victoria, BC.

Bealle-Statland, C. (1998) Stand structure and growth estimates for the Opax Mountain silvicultural systems trial. In *Managing the dry Douglas-fir forests of the Southern Interior: workshop proceedings April 29–30, 1997* (A. Vyse, C. Hollstedt & D. Huggard, eds): 136–148. [Working Paper 34/1998.] Research Branch, BC Ministry of Forests, Victoria, BC.

Bidartondo, M. I., Kreitzer, A. M., Pine, E. M. & Bruns, T. D. (2000) High root concentration and uneven ectomycorrhizal diversity near *Sarcodes sanguinea* (*Ericaceae*): a cheater that stimulates its victims? *American Journal of Botany* **87**: 1783–1788.

Bradbury, S. M. (1998) Ectomycorrhizas of lodgepole pine (*Pinus contorta*) seedlings originating from seed in southwestern Alberta cut blocks. *Canadian Journal of Botany* **76**: 213–217.

Brundrett, M. C. & Abbott, L. K. (1995) Mycorrhizal fungus propagules in the jarrah forest. II. Spatial variability in inoculum levels. *New Phytologist* **13**: 461–469.

Bruns, T. D., Szaro, T. M., Gardes, M., Cullings, K. W., Pan, J. J., Taylor, D. L., Horton, T. R., Kretzer, A., Garbelotto, M. & Li, Y. (1998) A sequence database for the identification of ectomycorrhizal basidiomycetes by phylogenetic analysis. *Molecular Ecology* **7**: 257–272.

Chambers, S. M., Sawyer, N. A. & Cairney, J. W. G. (1999) Molecular identification of co-occurring *Cortinarius* and *Dermocybe* species from southeastern Australian sclerophyll. *Mycorrhiza* **9**: 85–90.

Conn, C. & Dighton, J. (2000) Litter quality influences on decomposition, ectomycorrhizal community structure and mycorrhizal root surface acid phosphatase activity. *Soil Biology & Biochemistry* **32**: 489–496.

Dahlberg, A. (2001) Community ecology of ectomycorrhizal fungi: an advancing interdisciplinary field. *New Phytologist* **150**: 555–562.

Dahlberg, A., Jonsson, L. & Nylund, J.-E. (1997) Species diversity and distribution of biomass above and below ground among ectomycorrhizal fungi in an old-growth Norway spruce forest in south Sweden. *Canadian Journal of Botany* **75**: 1323–1335.

Danielson, R. M. (1991) Temporal changes and effects of amendments on the occurrence of sheathing (ecto-) mycorrhizas of conifers growing in oil sands tailings and coal spoil. *Agriculture Ecosystems & Environment* **35**: 261–281.

Durall, D. M., Jones, M. D., Wright, E. F., Koreger, P. & Coates, K. D. (1999) Species richness of ectomycorrhizal fungi in cutblocks of different sizes in the Interior Cedar-Hemlock forests of northwestern British Columbia: sporocarps and ectomycorrhizae. *Canadian Journal of Forest Research* **29**: 1322–1332.

Eberhardt, U., Walter, L. & Kottke, I. (1999) Molecular and morphological discrimination between *Tylospora fibrillose* and *Tylospora asterophora* mycorrhizae. *Canadian Journal of Botany* **77**: 11–21.

Egger, K. N. (1995) Molecular analysis of ectomycorrhizal fungal communities. *Canadian Journal of Botany* **73**: S1415–S1422.

Erland, S., Jonsson, T., Mahmood, S. & Finlay, R. D. (1999) Belowground ectomycorrhizal community structure in two *Picea abies* forests in southern Sweden. *Scandinavian Journal of Forest Research* **14**: 209–217.

Farmer, D. J. & Sylvia, D. M. (1998) Variation in the ribosomal DNA internal transcribed spacer of a diverse collection of ectomycorrhizal fungi. *Mycological Research* **102**: 859–865.

Fransson, P. M. A., Taylor, A. F. S. & Finlay, R. D. (2000) Effects of continuous optimal fertilization on belowground ectomycorrhizal community structure in a Norway spruce forest. *Tree Physiology* **20**: 599–606.

Gardes, M. & Bruns, T. D. (1993) ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**: 113–118.

Gardes, M. & Bruns, T. D. (1996) Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: above- and below-ground views. *Canadian Journal of Botany* **74**: 1572–1583.

Gardes, M., White, T. J., Fortin, J. A., Bruns, T. D. & Taylor, J. W. (1991) Identification of indigenous and introduced symbiotic fungi in ectomycorrhizae by amplification of nuclear and mitochondrial ribosomal DNA. *Canadian Journal of Botany* **69**: 180–190.

Ginns, J. & Lefebvre, M. N. L. (1993) *Lignicolous Corticioid Fungi* (*Basidiomycota*) *of North America*. American Phytopathological Society Press, St Paul, MN.

Goodman, D. M., Durall, D. M. & Trofymow, J. A. (1996) Describing morphology and anatomy. In *Concise Descriptions of*

North American Ectomycorrhizae (D. M. Goodman, D. M. Durall, J. A. Trofymow & S. M. Berch, eds): 3A.1–3A.5. Mycologue Publications, Victoria, BC.

Goodman, D. M., Durall, D. M., Trofymow, J. A. & Berch, S. M. (eds.) (1996–2000) *Concise Descriptions of North American Ectomycorrhizae*. Mycologue Publications, Victoria, BC.

Hagerman, S. M., Jones, M. D., Bradfield, G. E., Gillespie, M. & Durall, D. M. (1999a) Effects of clear-cut logging on the diversity and persistence of ectomycorrhizae at a subalpine forest. *Canadian Journal of Forest Research* **29**: 124–134.

Hagerman, S. M., Jones, M. D., Bradfield, G. E. & Sakakibara, S. M. (1999b) Ectomycorrhizal colonization of *Picea engelmannii* × *Picea glauca* seedlings planted across cut blocks of different sizes. *Canadian Journal of Forest Research* **29**: 1856–1870.

Hagerman, S. M., Sakakibara, S. M. & Durall, D. M. (2001) The potential for woody understory plants to provide refuge for ectomycorrhizal inoculum at an Interior Douglas-fir forest after clear-cut logging. *Canadian Journal of Forest Research* **31**: 711–721.

Haug, I. & Oberwinkler, F. (1987) Some distinctive types of spruce mycorrhizae. *Trees-Structure & Function* **1**: 172–188.

Henrion, B., Le Tacon, F. & Martin, F. (1992) Rapid identification of genetic variation of ectomycorrhizal fungi by amplification of ribosomal RNA genes. *New Phytologist* **122**: 289–298.

Horton, T. R. & Bruns, T. D. (1998) Multiple-host fungi are the most frequent and abundant ectomycorrhizal types in a mixed stand of Douglas-fir (*Pseudotsuga menziesii*) and bishop pine (*Pinus muricata*). *New Phytologist* **139**: 331–339.

Horton, T. R. & Bruns, T. D. (2001) The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. *Molecular Ecology* **10**: 1855–1877.

Ingleby, K., Mason, P. A., Last, F. T. & Fleming, L. V. (1990) *Identification of Ectomycorrhizas*. [ITE Research Publication No. 5.] HMSO, London.

Jones, M. D., Durall, D. M., Harniman, S. M. K., Classen, D. C. & Simard, S. W. (1997) Ectomycorrhizal diversity on *Betula papyrifera* and *Pseudotsuga menziesii* seedlings grown in the greenhouse or outplanted in single-species and mixed plots in southern British Columbia. *Canadian Journal of Forest Research* **27**: 1872–1889.

Jonsson, L., Dahlberg, A., Nilsson, M. C., Zackrisson, O. & Kårén, O. (1999a) Ectomycorrhizal fungal communities in late-successional Swedish boreal forests, and their composition following wildfire. *Molecular Ecology* **8**: 205–215.

Jonsson, L., Dahlberg, A., Nilsson, M.-C., Kårén, O. & Zackrisson, O. (1999b) Continuity of ectomycorrhizal fungi in self-regenerating boreal *Pinus sylvestris* forests studied by comparing mycobiont diversity on seedlings and mature trees. *New Phytologist* **142**: 151–162.

Kårén, O. & Nylund, J.-E. (1997) Effects of ammonium sulphate on the community structure and biomass of ectomycorrhizal fungi in a Norway spruce stand in southwestern Sweden. *Canadian Journal of Botany* **75**: 1628–1642.

Kårén, O., Högberg, N., Dahlberg, A., Jonsson, L. & Nylund, J.-E. (1997) Inter- and intraspecific variation in the ITS region of rDNA of ectomycorrhizal fungi in Fennoscandia as detected by endonuclease analysis. *New Phytologist* **136**: 313–325.

Kernaghan, G., Currah, R. S. & Bayer, R. J. (1997) Russulaceous ectomycorrhizae of *Abies lasiocarpa* and *Picea engelmannii*. *Canadian Journal of Botany* **75**: 1843–1850.

Kranabetter, J. M. & Wylie, T. (1998) Ectomycorrhizal community structures across forest openings on naturally regenerated western hemlock seedlings. *Canadian Journal of Botany* **76**: 189–196.

Lloyd, D. A., Angove, K., Hope, G. & Thompson, C. (1990) *A Guide to Site Identification and Interpretation for the Kamloops Forest Region*. [B.C. Ministry of Forests, Land Management Handbook No. 23.] Forestry Canada and British Columbia Ministry of Forests, Victoria, BC.

Mah, K., Tackaberry, L., Egger, K. N. & Massicotte, H. B. (2001) The impacts of broadcast burning after clear-cutting on the diversity of ectomycorrhizal fungi associated with hybrid spruce seedlings in central British Columbia. *Canadian Journal of Forest Research* **31**: 224–235.

Massicotte, H. B., Molina, R., Tackaberry, L. E., Smith, J. E. & Amaranthus, M. P. (1999) Diversity and host specificity of ectomycorrhizal fungi retrieved from three adjacent forest sites by five host species. *Canadian Journal of Botany* **77**: 1053–1076.

Mehmann, B., Egli, S., Braus, G. H. & Brunner, I. (1995) Coincidence between molecularly or morphologically classified ectomycorrhizal morphotypes and fruitbodies in a spruce forest. In *Biotechnology of Ectomycorrhizae – Molecular Approaches* (V. Stocchi, P. Bonfante & M. Nuti, eds): 41–52. Plenum Press, New York.

Nylund, J.-E., Dahlberg, A., Högberg, N., Kårén, O., Grip, K. & Jonsson, L. (1995) Methods for studying species composition of mycorrhizal fungal communities in ecological studies and environmental monitoring. In *Biotechnology of Ectomycorrhizae – Molecular Approaches* (V. Stocchi, P. Bonfante & M. Nuti, eds): 229–239. Plenum Press, New York.

Olsson, P. A., Münzenberger, B., Mahmood, S. & Erland, S. (2000) Molecular and anatomical evidence for a three-way association between *Pinus sylvestris* and the ectomycorrhizal fungi *Suillus bovinus* and *Gomphidius roseus*. *Mycological Research* **104**: 1372–1378.

Pritsch, K., Boyle, H., Munch, J. C. & Buscot, F. (1997) Characterization and identification of black alder ectomycorrhizas by PCR/RFLP analyses of the rDNA internal transcribed spacer (ITS). *New Phytologist* **137**: 357–369.

Read, D. J. (2000) Links between genetic and functional diversity – a bridge too far? *New Phytologist* **145**: 363–365.

Roth, D. R. & Fahey, T. J. (1998) The effects of acid precipitation and ozone on the ectomycorrhizae of red spruce saplings. *Water, Air & Soil Pollution* **103**: 263–276.

Rygiewicz, P. T., Martin, K. J. & Tuininga, A. R. (2000) Morphotype community structure of ectomycorrhizas on Douglas fir (*Pseudotsuga menziesii* Mirb. Franco) seedlings grown under elevated atmospheric CO_2 and temperature. *Oecologia* **124**: 299–308.

Simard, S. W., Perry, D. A., Jones, M. D., Myrold, D. D., Durall, D. M. & Molina, R. (1997) Net transfer of carbon between ectomycorrhizal tree species in the field. *Nature* **388**: 579–582.

Soil Classification Working Group (1998) *The Canadian System of Soil Classification*. [Agriculture and Agri-food Canada Publication No. 1646 (Revised).] NRC Research Press, Ottawa.

Stendell, E. R., Horton, T. R. & Bruns, T. D. (1999) Early effects of prescribed fire on the structure of the ectomycorrhizal fungus community in a Sierra Nevada ponderosa pine forest. *Mycological Research* **103**: 1353–1359.

Taylor, J. W., Jacobson, D. J., Kroken, S., Kasuga, T., Geiser, D. M., Hibbett, D. S. & Fisher, M. C. (2000) Phylogenetic species recognition and species concepts in fungi. *Fungal Genetics and Biology* **31**: 21–32.

van der Heijden, E. W., Vries, F. W. de. & Kuyper, T. W. (1999) Mycorrhizal associations of Salix repens L. communities in succession of dune ecosystems. I. Above-ground and below-ground views of ectomycorrhizal fungi in relation to soil chemistry. *Canadian Journal of Botany* **77**: 1821–1832.

Visser, S. (1995) Ectomycorrhizal fungal succession in jack pine stands following wildfire. *New Phytologist* **129**: 389–401.

Vrålstad, T., Fossheim, T. & Schumacher, T. (2000) *Piceirhiza biolorata* expression of the *Hymenoscyphus ericae* aggregate? *New Phytologist* **145**: 549–563.

Wallenda, T. & Read, D. J. (1999) Kinetics of amino acid uptake by ectomycorrhizal roots. *Plant, Cell & Environment* **22**: 179–187.

Wallender, H., Arnebrant, K., Östrand, F. & Kårén, O. (1997) Uptake of ^{15}N-labelled alanine, ammonium and nitrate in *Pinus sylvestris* L. ectomycorrhizae growing in forest soil treated with nitrogen, sulphur or lime. *Plant and Soil* **195**: 329–338.

White, T. J., Bruns, T. D., Lee, S. & Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for polygenetics. In *PCR Protocols: a guide to methods and applications*

(M. A. Innis, D. H. Gelgand, J. J. Sninsky & T. J. White, eds): 315–322. Academic Press, San Diego.

Zak, B. (1973) Classification of ectomycorrhizae. In *Ectomycorrhizae: their ecology and physiology* (G. C. Marks & T. T. Kozlowski, eds). Academic Press, New York.

Zhou, Z., Miwa, M., Matsuda, Y. & Hogetsu, T. (2001) Spatial distribution of the subterranean mycelia and ectomycorrhizae of *Suillus grevillei* genets. *Journal of Plant Research* **114**: 179–185.

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