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A molecular and phylogenetic analysis of the structure and specificity of *Alnus rubra* ectomycorrhizal assemblages

*Peter G. KENNEDY**, *Lee T. HILL*

Department of Biology, Lewis and Clark College, 0615 S.W. Palatine Hill Road, Portland, OR 97219, United States

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ABSTRACT

Ectomycorrhizal (EM) assemblages associated with *Alnus* spp. are often distinct in composition and richness from other host plants. To examine the EM assemblage associated with *A. rubra*, a common tree in western coastal North America, we sampled four *A. rubra* forests varying in age, management history, and geographic location in Oregon, USA. From the 364 EM root tip rDNA ITS sequences, we found a total of 14 EM taxa. The five most abundant taxa, *Tomentella* sp. 3, *Alnicola escharoides*, *Tomentella* sp. 1, *Lactarius cf. obscuratus*, and *Alpova diplophloeus*, represented 80 % of the samples and were present at all four sites. Assemblage structure differed significantly among young managed sites and older unmanaged sites but not by geographic location. The younger managed sites had higher tree density, *Frankia* frequency, and soil nitrogen than older unmanaged sites. Phylogenetic analyses revealed that *Alnus*-associated EM congeners were not closely related, indicating the distinct nature of *Alnus* EM assemblages is not due to a unique co-evolutionary history.

Introduction

Ectomycorrhizal (EM) fungal assemblages are affected by a wide range of factors including dispersal (Peay *et al.* 2007), environmental conditions (Erland & Taylor 2002) and species interactions (Koide *et al.* 2005). Due to the obligate nature of the EM symbiosis, the environmental conditions influencing EM assemblages include both abiotic (e.g. temperature, pH) and biotic components (e.g. host plant composition). A number of EM species have been shown to associate with a limited range of host plants, while many others appear to have little host specificity (Molina *et al.* 1992). Although absolute host specificity is relatively uncommon among EM fungi (Horton & Bruns 1998; Kennedy *et al.* 2003), differences in host preference can significantly influence EM assemblage structure, particularly in comparisons among more distantly related hosts (Ishida *et al.* 2007; Tedersoo *et al.* 2008; Smith *et al.* 2009).

Much of the early work about host effects on EM assemblages focused on the genus *Alnus* (Neal *et al.* 1968; Molina 1979; Molina 1981). Plants in this genus are involved in tri- or tetra-partite symbioses with nitrogen-fixing *Frankia* bacteria, EM fungi, and arbuscular mycorrhizal fungi (Chatarpaul *et al.* 1989; Molina *et al.* 1994). Along with *Frankia* bacteria, EM fungi co-dominate the roots of older *Alnus* individuals and play a significant role in nutrient acquisition and growth (Mejstrik & Benecke 1969; Molina *et al.* 1994; Yamanaka *et al.* 2003). Relative to other well-studied plant genera, *Alnus* hosts a low number of EM species, with only 50 species reported across the entire genus (Molina *et al.* 1994). The pattern of lower EM richness on *Alnus* has been seen in both observational and experimental studies. Molina (1979) found that only 4 of 28 EM species formed ectomycorrhizas with *Alnus rubra* using pure-culture syntheses. Similar patterns were observed in other experiments (Molina 1981; Molina & Trappe 1982) and *Alnus*

* *Corresponding author*. Tel.: +1 503 768 7509; fax: +1 503 768 7568.
E-mail address: pkennedy@lclark.edu (P.G. Kennedy).
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forests generally have lower sporocarp production and a greater proportion of host-specific species than adjacent forests (J.M. Trappe, pers. com.). Molecular-based studies have confirmed that *Alnus* EM assemblages are less diverse than those found on other hosts, although many of the same genera (e.g. *Tomentella*, *Cortinarius*, *Lactarius*) can dominate *Alnus* and other EM host forests (Pritsch *et al.* 1997; Beccera *et al.* 2005; Tedersoo *et al.* 2009).

While the distinct nature of *Alnus* EM assemblages is widely recognized, the mechanism(s) determining their lower richness and higher proportion of host-specific species are less clearly understood. The EM assemblages associated with the closely related host genus, *Betula*, are more diverse and less host-specific (Jones *et al.* 1997; DeBellis *et al.* 2006), indicating the unique composition of *Alnus* EM assemblages is not characteristic of this host plant family. Molina *et al.* (1994) suggested that *Alnus*-associated EM fungi may share a close co-evolutionary history with this host genus. If this is true, then one could expect *Alnus*-associated EM species to form closely related monophyletic clades within their respective genera due to a unique evolutionary relationship with *Alnus*. Alternatively, if the congeners of *Alnus*-associated EM species are not closely related, it would suggest that other factors play a more important role in determining the composition of *Alnus* EM assemblages. Support for the greater importance of non-evolutionary factors was recently demonstrated by Moreau *et al.* (2006), who found that *Alnus*-associated *Alnicola* species did not form a monophyletic clade within the genus. Whether this pattern is representative of other *Alnus*-associated EM genera, however, is not clear.

Along the Pacific coast of the United States and Canada, *A. rubra* (Red Alder) is the most abundant deciduous tree species in many forests (Hibbs *et al.* 1994). It is commonly found in geologically young, disturbed, and riparian areas and plays an important role in successional dynamics in this region (Shainsky & Radosevich 1992; Hibbs *et al.* 1994). A number of sporocarp and root tip morphotype studies have been conducted on the EM fungi associated with *A. rubra* (Neal *et al.* 1968; Molina 1979; Molina 1981; Miller *et al.* 1991). From these studies, it appears that *A. rubra* EM colonization is high and that assemblages are dominated by two *Alnus*-specific EM species, *Alpova diplophloeus* and *Lactarius obscuratus*. Only 11 EM species are known or suspected to associate with *A. rubra* across its geographic range, which is much lower than that of co-occurring EM hosts (e.g. *Pseudotsuga menziesii* may associate with up to 2 000 EM species (Trappe & Fogel 1977)). Host specificity of *A. rubra* EM assemblages was experimentally demonstrated by Miller *et al.* (1992), who found little overlap in the morphotypes of EM fungi colonizing *A. rubra* and *P. menziesii* seedlings grown in the same soils collected from different forest successional stages.

Despite a considerable history of research about the EM fungi associated with *A. rubra*, no studies have examined EM assemblages in *A. rubra* forests using molecular methods, which typically capture greater species diversity than sporocarp or root tip morphotype-based methods (Peay *et al.* 2008). In addition, sequence-based molecular methods are a prerequisite for examining phylogenetic relationships among *Alnus*-associated EM species. To assess the composition and richness of *A. rubra* EM assemblages, we sampled four *A. rubra* forests varying in age, management history, and geographic location and compared EM assemblages using sequence-based analyses of the rDNA ITS region. Specifically, we addressed three questions: (1) what is the composition and structure of EM assemblages in *A. rubra* forests?; (2) is the structure of *A. rubra* EM assemblages affected by either site host age and management history or geographic location?; and (3) within genera, how are the EM fungi associated with *Alnus* species related to one another?

Methods

Site characteristics

Four sites in western Oregon, U.S.A. were chosen for sampling. Two of the sites are part of a long-term research project examining the effects of different forest management practices on *A. rubra* growth (see the Hardwood Silvicultural Cooperative (HSC) website for details, http://www.cof.orst.edu/coops/hsc). The HSC sites in this study, Toledo (HSC 2208) and Thompson Cat (HSC 5203), were established in 1992. Prior to the HSC project, the sites were second-growth coniferous forests that were clear-cut the year before *A. rubra* monocultures were planted. Thompson Cat was burned as part of the site planting preparation, Toledo was not. *A. rubra* seedlings were planted from nursery stock (Brooks Tree Farm, Brooks, OR) during the beginning of their second year of growth. Seedling EM and *Frankia* nodule status at the time of planting was not assessed, but nursery fumigation practices indicate colonization was unlikely (Brooks Nursery, pers. com.). Seedlings in the areas sampled at each site were planted at a density of 2 967 ha^{-1}, which is comparable to that seen in naturally regenerating *A. rubra* stands (A. Bluhm, pers. com.). Aside from site preparation and tree density, no other forest management treatments were applied. The two HSC sites were located on the west sides of Oregon's coast range and Cascade mountains, respectively. The third site, Fox Creek, was located in the Tillamook State Forest on the western side of the coast range mountains. This site was a naturally regenerating mature *A. rubra* stand approximately 70 yr in age (Tillamook Ranger District, pers. com.). The fourth site, Mt. Hood, was located in Mt. Hood National forest in the western Cascade mountains. Like Fox Creek, this site was a naturally regenerating approximately 60 yr old stand (Clackamas Ranger District, pers. com.). Understory vegetation at all sites was similar and dominated by the non-EM host plants *Polystichum munitum* and *Rubus ursinus*. The only other EM host was *Corylus cornuta*, which was present in very low abundance at the Fox Creek site. Additional site details are listed in Table 1.

Sampling design

At each site, we located a 900 m^2 area for EM root tip sampling. Twenty ~15 cm^3 soil cores were taken from within 2 m of a randomly located *A. rubra* individual. An effort was made to take cores in areas where *Frankia* colonized roots were present to confirm host root identity. This was accomplished by removing the litter layer and first lightly raking in the vicinity

Table 1 – Geographic, forest, soil, and climate information for the four study sites

Site	Location (lat, long)	Geographic region	Altitude (m)	Mean precipitation (mm/yr)	Mean temperature (°C)
Mt. Hood	N45′09.091″ W122′08.496″	Cascade	556	1973	9
Fox Creek	N45′34.007″ W123′34.119″	Coast range	154	2138	10.1
Thompson Cat	N45′29.514″ W122′12.177″	Cascade	391	1983	9.7
Toledo	N44′37.700″ W123′54.659″	Coast range	38	2312	10.7
Site	Soil series	Forest age (yr)	Tree density (ha)	Nodule Frequency[a]	Total soil N (%)
Mt. Hood	Not available	~60	400	5/10	0.69 (0.24) ab
Fox Creek	Alic-Hapludands complex	16	360	3/10	0.45 (0.16) b
Thompson Cat	Aschoff cobbly loam	16	2967	9/10	0.86 (0.26) a
Toledo	Toloyana-Reedsport complex	~70	2967	7/10	0.95 (0.27) a

[a] Nodule frequency is calculated as the number of cores in which nodules were present out of 10.

of each randomly located tree. *Frankia* nodules are conspicuously colored and visible to the naked eye, so once a host root with a nodule was confirmed, the core was taken directly around it. Cores were transported back to the laboratory on ice and maintained as such until processing. Within 10 d of sampling, cores were individually washed over a 0.355 mm sieve and all EM root tips were removed. EM root tips were distinguished from non-EM root tips based on standard morphological characters (e.g. swelling, colour, branching morphology, etc.) using a 40× dissecting microscope. No morphotyping was done to separate EM tips prior to sampling for molecular analyses. For each core, 10 EM tips were randomly selected from the pool of all EM tips removed, placed into CTAB buffer (2 % CTAB, 0.1 M Tris pH 8.0, 1 M NaCl, 0.02 M EDTA), and stored at −20 °C. Three cores were not included in further analyses due to a lack of EM root tips, a core processing error, and unsuccessful PCR amplification. Sporocarps of all putative EM species were collected from sites during root tip sampling, brought back to the laboratory for identification, and dried for long-term storage.

Soils at each site were also assessed for *Frankia* nodule frequency and total soil nitrogen. To quantify *Frankia* frequency, 10 randomly located 25 × 25 × 10 cm soil cores were collected from the same area sampled for EM root tips. In the laboratory, each sample was washed over a 2 mm sieve, searched for nodules, and scored as nodules present or absent. An additional 10 randomly located soil cores from each site were taken from the top 10 cm of mineral soil to assess total soil nitrogen. Cores were dry-sieved through a 2 mm sieve, dried at 80 °C for 48 hr, and sent to the Central Analytical Laboratory at Oregon State University for analysis with a Leco CN-2000 Macro Analyzer.

Molecular protocols

Total genomic DNA for EM root tips and sporocarps was extracted using the REDExtract-N-Amp Plant PCR kit (Sigma–Aldrich, St. Louis, MO). Individual root tips or sections of sporocarp tissue were added to 20 μl of extraction solution and cycled at 65 °C for 10 min, followed by 95 °C for 10 min. After incubation, 40 μl of neutralization solution was added and DNA extracts were stored at −20 °C. PCR was done in 25 μl reactions using standard cycling parameters for the fungal specific primer combination ITS1f and ITS4 (Gardes & Bruns 1993). For mixed samples, which represented a very small proportion of the total tips analyzed, we used the ITS1f and ITS4b primer combination to eliminate possible contamination from ascomycetous root endophytes (Gardes & Bruns 1993). Initial PCR success varied among cores (ranging from 1 to 10), but all cores had at least 5 successfully amplified EM root tips after the two rounds of PCR. Preliminary analyses showed that sequencing all 10 EM tips per core did not significantly change the within-core species richness because cores generally tended to have only one or two species present, which were captured in 5 EM tips. As such, the PCR products from only 5 EM tips per core were cleaned using 2 μl of ExoSAP IT (USB Corp., Cleveland, OH) with 10 μl of DNA and cycled at 37 °C for 45 min, followed by 80 °C for 15 min. DNA sequencing was performed on a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA) at the Genomic Analysis and Technology Core Facility at the University of Arizona, USA. Sequence chromatograms were visually confirmed and contiguous sequences were assembled using Sequencher 4.8 (Gene Codes Corp., Ann Arbor, MI).

Identification of EM taxa

Taxa were identified using a combination of morphological and molecular techniques. Sporocarps were identified to the lowest taxonomic level possible using standard morphological keys. Sequences from root tips and sporocarps were grouped across all samples at ≥97 % similarity, a threshold shown to distinguish the majority of EM species (Peay *et al.* 2008). Consensus sequences of each taxon were then compared with the NCBI and UNITE databases. Names for taxa without fully identified fruitbodies were designated based upon the taxonomic level supported from the database queries. Only samples from unambiguous EM lineages were included in the final analyses. A randomly selected sequence from each EM taxon was submitted to GenBank under the accession numbers: GQ398240–GQ398253.

Identification of host

For EM taxa that occurred in low abundance (in a single core or at a single site), we confirmed host root identity by amplifying a portion of host DNA using the *Alnus*-specific primers

ITS1-plant and ITS-*Alnus*2 (Navarro *et al.* 2003). Samples without a positive amplicon corresponding to the same size as that from a surface sterilized *Alnus* leaf DNA sample were excluded from all further analyses.

Phylogenetic analyses

To examine the relationships among *Alnus* and non-*Alnus*-associated EM taxa, we conducted a series of phylogenetic analyses. We focused on three genera, *Tomentella*, *Lactarius*, and *Cortinarius*, due to their abundance in our study system, availability of sequences with detailed host information, and access to pre-constructed alignments. Sequences were collected from other coastal western North American forests where *A. rubra* co-occurs (Kennedy *et al.* 2003; Cline *et al.* 2005; Horton *et al.* 2005; Peay *et al.* 2007), other studies of *Alnus* EM assemblages (Beccera *et al.* 2005; Tedersoo *et al.* 2009) as well as genus-specific studies (*Tomentella*, Koljalg *et al.* 2000; *Cortinarius*, Liu *et al.* 1997; Seidl 2000; Peintner *et al.* 2001; Peintner *et al.* 2003) and the UNITE database (Koljalg *et al.* 2005).

For the *Cortinarius* phylogenetic analysis, we focused on the subgenus Telemonia based on the BLAST affinities of the two *A. rubra*-associated *Cortinarius* taxa. Additional *Alnus*-associated *Cortinarius* sequences were generated from sporocarp collections at the University of Washington herbarium. For the *Lactarius* phylogenetic analysis, neighbor joining and maximum parsimony analyses were first conducted to place our *A. rubra*-associated *Lactarius* taxon within the larger *Lactarius* phylogenetic analysis of Geml *et al.* (2009). Once the location of the *A. rubra*-associated *Lactarius* taxon was established (both analyses were concordant) only other *Lactarius* taxa from the clade into which it fell were included in the final analysis. For the *Tomentella* phylogenetic analysis, all of the EM taxa from Koljalg *et al.* (2000) with host information were included as well as a broad range of additional host identified *Tomentella* taxa from the UNITE database.

Alignments were done in CLUSTALW using the profile alignment option to match pre-constructed alignments of each genus (*Tomentella*, S. Bergemann, unpublished data; *Cortinarius*, Peintner *et al.* 2003; *Lactarius*, Geml *et al.* 2009). Maximum likelihood and Bayesian analyses were conducted using PhyML 3.0 (Guindon & Gascuel 2003) and Mr. Bayes v3.1.2 (Ronquist & Huelsenbeck 2003), respectively. Modeltest (Posada & Crandell 1998) using the Akaike Information Criterion determined that GTR + I + G model of sequence evolution fit the data best for all three genera. ML analyses were conducted on the Montepellier bioinformatics platform (http://www.atgc-montpellier.fr/phyml/) using the following settings: substitution model GTR, proportion of invariable sites fixed, gamma parameter estimated, number of substitution categories = 4, starting tree BIONJ, type of tree improvement NNI, optimized topology and branch lengths, and SH-like aLRT scores calculates. The Bayesian analyses included 10 million generations of two simultaneous runs. The average standard deviation of split frequencies at the end of the runs was 0.019 for *Tomentella* and <0.01 for both *Cortinarius* and *Lactarius*. Each run included four Markov Chain Monte Carlo (MCMC) chains and trees were constructed following a visual determined burn-in of 10 %.

Statistical analyses

EstimateS was used to estimate EM taxa richness and calculate accumulation curves at each site (Colwell 2005). Estimates were based on 500 randomizations of sample order without replacement. The non-parametric Chao2 estimator was used based on the recommendations of Brose *et al.* (2003) because the observed number of species is often the least accurate estimator of true species richness (Colwell & Coddington 1994). PRIMER 5 was used to compare EM assemblages among sites (Clarke & Warwick 2001). A presence/absence taxa matrix was first created using each core at all sites and then calculated the Bray-Curtis similarity among cores. A one-way analysis of similarity (ANOSIM) was used to test for significant differences in assemblage structure among sites followed by pair-wise comparisons based on age/management history or geographic location. To account for multiple tests, the significance level was adjusted to $p \leq 0.017$ using a Bonferroni correction. Total soil nitrogen levels were compared among sites using a one-way ANOVA. Prior to running the ANOVA, the variances among sites were confirmed to be homogenous based on visual inspection of residual plots. A post-hoc Tukey test was used for specific comparisons of site means. The latter analyses were conducted in JMP 5.01 (SAS Institute Inc, Cary, NC) and considered significant at $p \leq 0.05$.

Results

Of the 385 root tips sequenced, 377 were successfully identified and 364 belonged to EM taxa. A total of 14 taxa were encountered across all sites (Table 2). Five taxa, *Tomentella* sp. 3, *Alnicola escharoides*, *Lactarius cf. obscuratus*, *Tomentella* sp. 1, and *Alpova diplophloeus*, accounted for 80 % of the EM tips sampled. The five dominant taxa were present at all four sites, while 5 EM taxa were unique to single sites (Table 2). Although taxa richness varied among sites (range 6–13), the Chao2 and rarefaction curves reached similar plateaus at each site, indicating that the sampling effort was sufficient to capture local site richness (Fig 1).

Comparing across the four sites, there was a significant difference in assemblage structure ($r = 0.10$, $p = 0.001$). This difference was significant when sites were grouped by site age/management history (i.e. younger managed *vs.* older unmanaged) ($r = 0.05$, $p = 0.015$), but not when sites were grouped by geographic location ($r = 0.02$, $p = 0.148$). The younger managed sites had considerably higher tree density as well as higher *Frankia* frequency than the older unmanaged sites (Table 1). In addition, total % soil nitrogen also varied across sites, being significantly higher at the two younger managed sites than at Fox creek (an older managed site), with Mt. Hood (an older managed site) being intermediate (Table 1).

Phylogenetic analyses showed that *Alnus*-associated taxa within the three genera examined were not monophyletic. Bayesian and maximum likelihood trees had similar topologies (Figs 2–4) and most clades were supported by high posterior probabilities and bootstrap values in all three analyses. For the *Tomentella* analysis, there were a total of seven independent clades which contained *Alnus*-associated taxa

Table 2 – *Alnus rubra* ectomycorrhizal (EM) taxa UNITE and GenBank similarity and abundance across four study sites

EM taxa	GenBank #	UNITE				BLASTn				Mt. Hood	Fox Creek	Thompson Cat	Toledo
		Closest match	UNITE #	% Match	# of bases	Closest match	GenBank #	% Match	# of bases	Tips (cores)	Tips (cores)	Tips (cores)	Tips (cores)
Alnicola escharoides	GQ398240	*Hebeloma erumpens*	UDB003193	92	629/682	*Naucoria escharoides*	AY900081	100	654/654	17 (9)	30 (9)	8 (5)	11 (3)
Alnicola sp. 1	GQ398241	*Hebeloma velutipes*	UDB000022	91	396/432	Uncultured EM	FM993252	99	426/430	2 (1)	0 (0)	0 (0)	0 (0)
Alpova diplophloeus	GQ398242	*Melanogaster variegatus*	UDB001487	90	343/380	*Alpova diplophloeus*	DQ989496	100	748/748	4 (2)	5 (1)	15 (6)	9 (4)
Cortinarius sp. 1	GQ398243	*Cortinarius rubrovioleipes*	UDB001453	95	556/583	*Cortinarius boulderensis*	FJ717500	95	552/577	8 (4)	4 (2)	7 (3)	0 (0)
Cortinarius sp. 2	GQ398244	*Cortinarius bibulus*	UDB002795	96	550/572	Uncultured EM	FM993205	99	561/562	3 (2)	3 (2)	0 (0)	0 (0)
Inocybe sp. 1	GQ398245	*Inocybe lanuginosa*	UDB002391	94	245/258	Uncultured EM	EF634111	87	577/663	0 (0)	0 (0)	0 (0)	9 (3)
Lactarius cf. obscuratus	GQ398246	*Lactarius obscuratus*	UDB002512	99	743/745	Uncultured EM	FM993233	99	697/702	17 (6)	23 (6)	8 (4)	10 (3)
Tomentella sp. 1	GQ398247	*Tomentella sublilacina*	UDB002972	99	631/635	Thelephorales	DQ195590	99	640/644	4 (2)	9 (4)	12 (6)	30 (8)
Tomentella sp. 2	GQ398248	*Tomentella sublilacina*	UDB002972	95	610/635	Uncultured EM	FM993226	98	627/635	3 (1)	1 (1)	0 (0)	10 (4)
Tomentella sp. 3	GQ398249	*Tomentella ellisii*	UDB000225	98	547/553	Uncultured EM	FM993262	99	633/637	10 (4)	22 (8)	45 (15)	9 (4)
Tomentella sp. 4	GQ398250	*Tomentella botryoides*	UDB000255	91	537/584	Uncultured EM	FM993148	98	621/629	6 (4)	0 (0)	0 (0)	1 (1)
Tomentella sp. 5	GQ398251	*Tomentella stuposa*	UDB002428	96	608/632	Uncultured EM	FM993236	99	637/639	3 (2)	0 (0)	0 (0)	0 (0)
Pseudotomentella sp. 1	GQ398252	*Pseudotomentella tristis*	UDB000029	99	650/654	Uncultured EM	FJ188355	99	652/656	2 (1)	1 (1)	0 (0)	0 (0)
Xerocomus sp. 1	GQ398253	*Xerocomus porosporus*	AY372288	92	608/654	*Xerocomus dryophila*	AY372288	95	659/687	3 (1)	0 (0)	0 (0)	0 (0)

Fig 1 – Ectomycorrhizal (EM) taxon accumulation curves at the four study sites. (A) Curves based on the Chao2 species richness estimator; (B) curves based on the observed number of taxa. Error bars represent one standard deviation. Site abbreviations are: FC = Fox Creek, MH = Mount Hood, TC = Thompson Cat, TO = Toledo.

(Fig 2). *Tomentella* sp. 1 clustered with a large clade of other *Alnus*-associated taxa, but the other four *A. rubra*-associated *Tomentella* taxa grouped more closely with taxa from other hosts. For the *Cortinarius* analysis, there were three independent clades which contained *Alnus*-associated taxa, with both of the *A. rubra*-associated *Cortinarius* taxa falling into the same clade (Fig 3). Similarly, the *Lactarius* analysis had three independent clades of *Alnus*-associated taxa. The *A. rubra*-associated *Lactarius cf. obscuratus* clustered closely with two European *Alnus*-associated taxa, *L. cyathuliformis* and an unindentified *Lactarius* EM sample (Fig 4).

Discussion

The richness and composition of the *A. rubra* EM assemblage corresponds well with that seen in other *Alnus* EM studies (Pritsch *et al.* 1997; Beccera *et al.* 2005; Tedersoo *et al.* 2009). In general, *Alnus* EM assemblages appear to have consistently lower taxa richness and unique composition compared to EM assemblages present on other host plants. In *A. rubra* forests, the EM assemblage is species poor compared to co-occuring EM hosts (Horton *et al.* 2005) and there is a suite of host-specific species (*Alpova diplophloeus*, *Lactarius cf. obscuratus*,

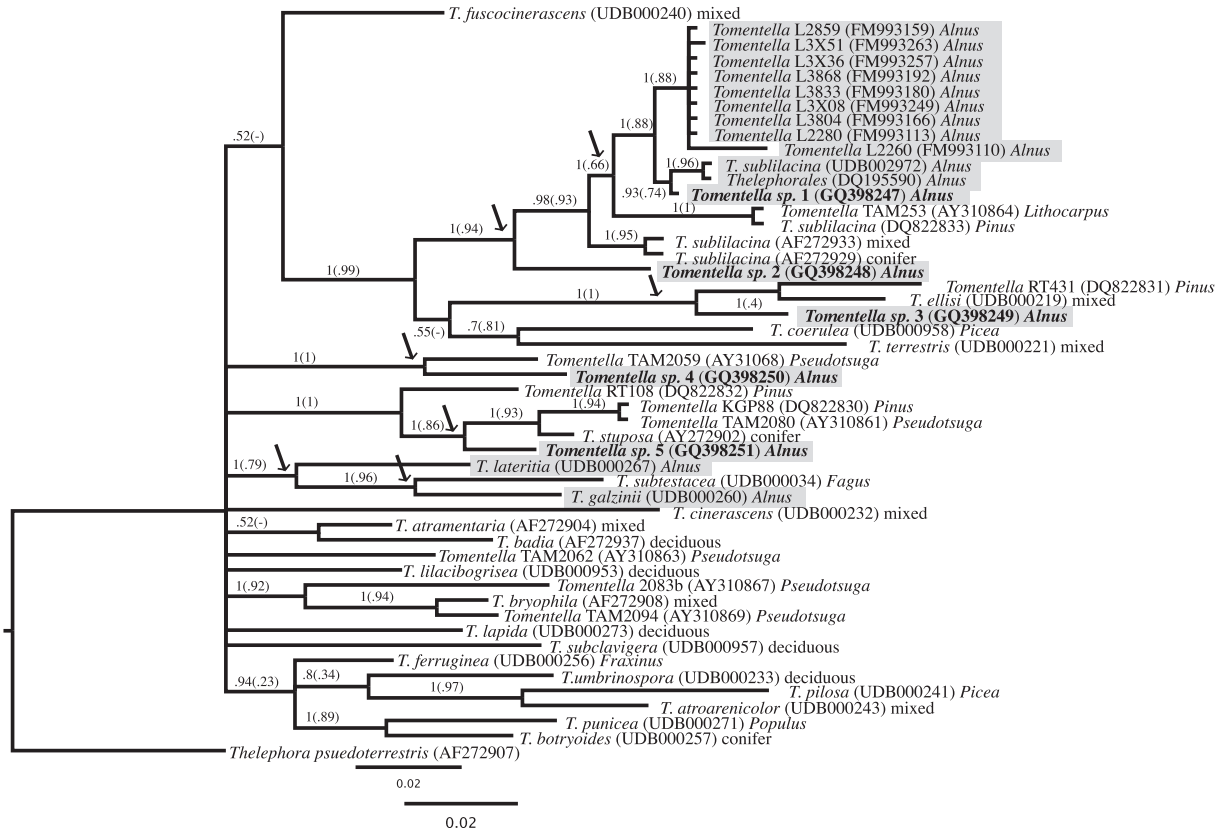

Fig 2 – Phylogenetic reconstruction of 50 *Alnus* and non-*Alnus*-associated *Tomentella* taxa based on rDNA ITS sequences. Nodes are labeled with posterior probabilities from Bayesian analysis and, in parentheses, bootstrap support values from maximum likelihood analysis. Dashes indicate that branch was not present in the maximum likelihood analysis. Taxa are coded by species epiphet, unique identifier (GenBank number, UNITE number, or collection number), and the type of host plant from which the sequence was obtained. Nodes with *Alnus*-associated *Tomentella* taxa are designated in gray boxes, with the *A. rubra*-associated *Tomentella* taxa in bold. Arrows indicate the number of clades in which *Alnus*-associated taxa are present. *Thelephora pseudoterrestris* was designated as the outgroup following Koljalg *et al.* (2000).

Alnicola escharoides) that are a major part of the *A. rubra* EM assemblages. There were both similarities and differences between the EM assemblage identified here and that based on morphotype-based analyses (Miller *et al.* 1991; Miller *et al.* 1992). Both methods showed similar levels of taxa richness, but the dominance and diversity of *Tomentella* taxa was not previously recognized with morphotyping. A similar pattern of high *Tomentella* abundance and diversity was observed in a recent molecular analysis of *Alnus* EM assemblages in Europe (Tedersoo *et al.* 2009). *Alnicola* spp., which were suspected *A. rubra* EM associates by Miller *et al.* (1991), were confirmed to be a significant component of *A. rubra* assemblages, particularly in older forests. Miller *et al.* (1991) identified three additional *A. rubra*-associated EM taxa (*Laccaria laccata*, *Paxillus involutus*, and *Hebeloma crustiliforme*) that were not encountered in the present study. Given the saturated taxon accumulation curves at all four sites, it appears that those taxa are not regularly associated with *A. rubra*, although *Alnus*-associated *Paxillus* and *Hebeloma* species have been found in other studies (Molina 1979; Tedersoo *et al.* 2009).

EM assemblage richness was not equally distributed among sites, but there was no clear pattern associated with site host age/management history or geographic location. In contrast, there was a significant shift in assemblage structure (i.e. composition, richness, and abundance) across the four sites associated with site host age/management history. A number of environmental variables also correlated with the observed assemblage changes, including tree density, *Frankia* frequency, and % total soil nitrogen (Table 1). Given the confounded nature of our sampling (i.e. age and management history are not independent) and the correlational nature of the study, we cannot determine which of these factors are most important. It is clear, however, that differing environmental conditions do impact the structure of *A. rubra* EM assemblages. Strong environment-associated assemblage effects have been previously observed in other *Alnus* EM studies. Beccera *et al.* (2005) found significant correlations between total soil nitrogen, phosphorus, and pH and the distribution of 5 of the 9 EM taxa associated with *A. acuminata*. Interestingly, the *Lactarius* and *Tomentella* taxa in the present study showed opposite relationships with respect to total soil nitrogen and abundance compared to their congeners in Beccera *et al.* (2005), suggesting genus-level comparisons among *Alnus* EM taxa should be made with caution. Tedersoo *et al.* (2009) determined that soil pH and total organic matter best explained variation in the structure of *A. glutinosa* and

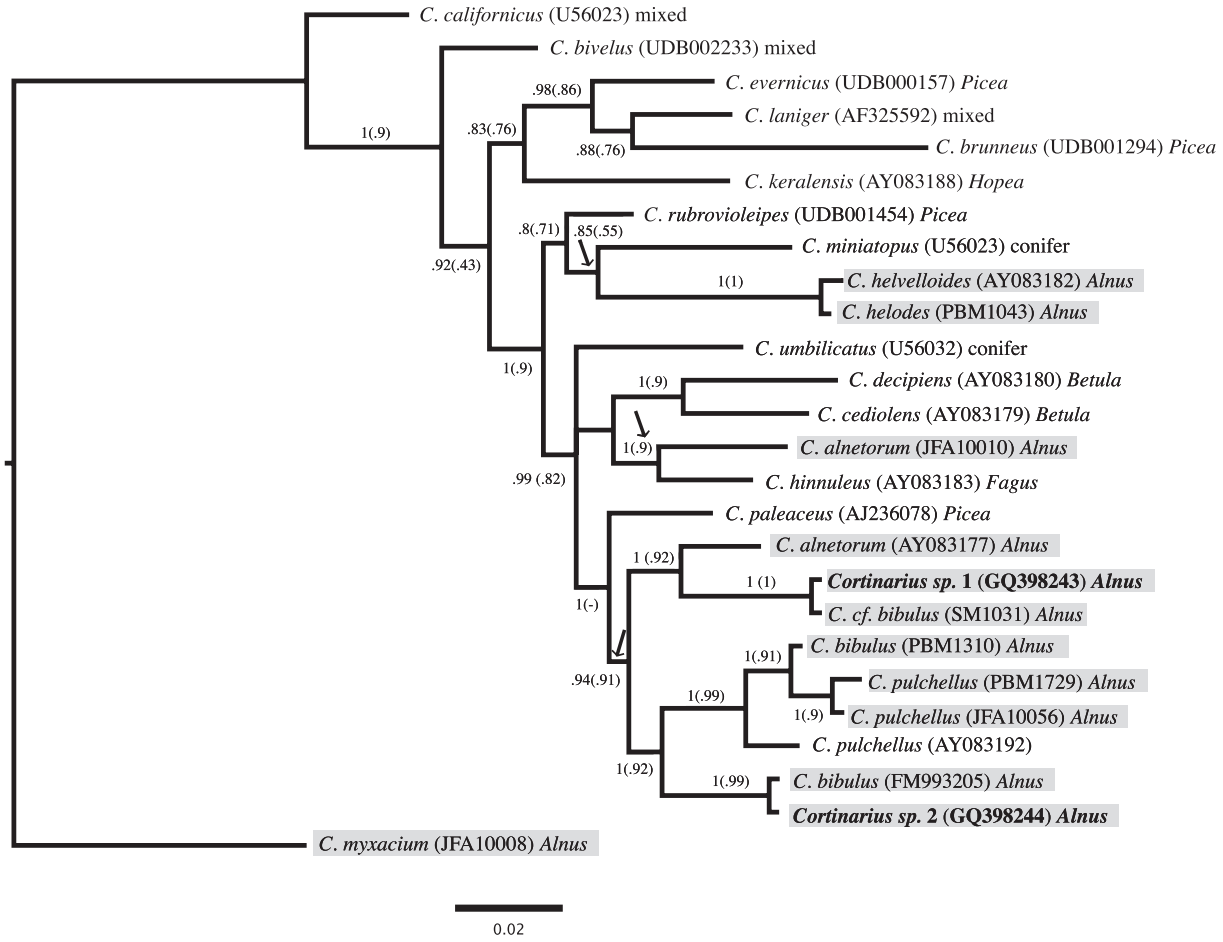

Fig 3 – Phylogenetic reconstruction of 27 *Alnus* and non-*Alnus*-associated *Cortinarius* subgenus Telemonia taxa based on rDNA ITS sequences. Nodes are labeled with posterior probabilities from Bayesian analysis and, in parentheses, bootstrap support values from maximum parsimony analysis. Taxa are coded by species epiphet, unique identifier (GenBank number, UNITE number, or collection number), and the type of host plant from which the sequence was obtained. Nodes with *Alnus*-associated *Cortinarius* taxa are designated in gray boxes, with the *A. rubra*-associated *Cortinarius* taxa in bold. Arrows indicate the number of clades in which *Alnus*-associated taxa are present. *Cortinarius myxacium*, which belongs to a different *Cortinarius* subgenus, was designated as the outgroup.

A. incana EM assemblages, further supporting the importance of soil parameters in determining local assemblage structure.

The phylogenetic analyses conducted on *Tomentella*, *Cortinarius*, and *Lactarius* all clearly demonstrated that *Alnus*-associated congeners are not each other's closest relatives. While these analyses are limited by (1) a lack of complete sampling of EM taxa from each group and (2) reliance on a single gene region, the consistency of these results strongly suggests that *Alnus*-associated EM species do not share a unique evolutionary history with this host genus. Additional support for this pattern has also been shown by Moreau *et al.* (2006), who found that *Alnus*-associated *Alnicola* species were present in multiple independent clades of ITS/LSU-based phylogenetic analyses. These combined findings have significant implications for understanding the distinct nature of *Alnus* EM assemblages. Rather than being the product of unique co-evolutionary relationships, it appears that other factors are more important in controlling the composition and structure of *Alnus* EM assemblages.

We believe the dominant factor is likely to be the presence of *Frankia* bacteria because they alter the soil and host environment in two significant ways. First, the abundance of nitrogen added to *Alnus* forests by *Frankia* (Bormann *et al.* 1994) is likely to favour EM fungi that can tolerate higher soil nitrogen conditions. A number of previous studies have shown that EM species have varying tolerances for different nitrogen levels (Lilleskov *et al.* 2002a; Cajsa *et al.* 2008) and that EM assemblages often show shifts in composition in nitrogen amended sites (Peter *et al.* 2001; Lilleskov *et al.* 2002b; Avis *et al.* 2003) and along natural soil nitrogen gradients (Toljander *et al.* 2006; Kranabetter *et al.* 2009). These findings from other forests suggest that higher soil nitrogen levels may provide an important environmental filter on *Alnus* EM assemblage composition. The abundance of nitrogen directly provided to *Alnus* individuals by *Frankia* may also influence host preference for EM fungi with varying nutrient uptake abilities. *Alnus* individuals colonized by *Frankia* (100 % in *A. rubra* forests) may prefer to associate with EM taxa with greater uptake of nutrients other than nitrogen because that requirement is already met by the other symbiont. There has been little experimental evidence examining the nutrient uptake abilities of *Alnus*-associated EM fungi

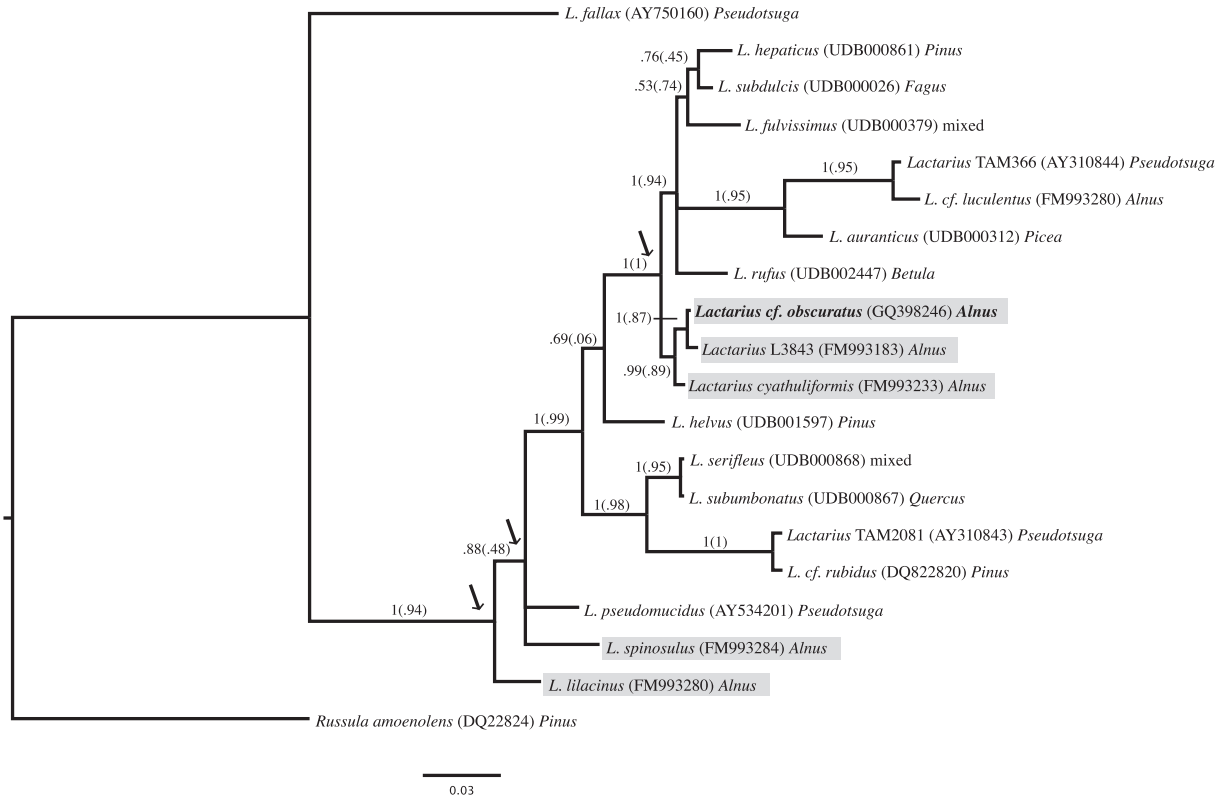

Fig 4 – Phylogenetic reconstruction of 19 *Alnus* and non-*Alnus*-associated *Lactarius* taxa based on rDNA ITS sequences. Nodes are labeled with posterior probabilities from Bayesian analysis and, in parentheses, bootstrap support values from maximum parsimony analysis. Taxa are coded by species epiphet, unique identifier (GenBank number or UNITE number) and the type of host plant from which the sequence was obtained. Nodes with *Alnus*-associated *Lactarius* taxa are designated in gray boxes, with the *A. rubra*-associated *Lactarius* taxon in bold. Arrows indicate the number of clades in which *Alnus*-associated taxa are present. *Russula amoenolens* was designated as the outgroup.

due to the challenges associated with culturing many of the dominant taxa, but recently developed culture-independent enzymatic assays (Courty *et al.* 2005) hold great promise in addressing this hypothesis.

Our results support the consensus that *Alnus* EM assemblages are distinct from those found on other EM hosts, but suggest that factors other than evolutionary history are responsible for their unique composition. Future manipulative studies involving both *Frankia* and multiple EM fungi would help in resolving the specific role that *Frankia* play in EM–host interactions. Thus far, experiments have only examined the response of *Alpova* spp. to *Frankia* (Koo 1989; Yamanaka *et al.* 2003), and additional studies targeted at the whole assemblage level are needed. Similarly, studies focused on mixed forests would help determine the specificity of *Alnus* EM assemblages in natural settings. Interestingly, Tedersoo *et al.* (2009) found that *Alnus* EM host specificity differed between fungal phyla, with only ascomycetes co-occuring on other hosts. The phylogenetic analyses presented here, however, suggest that multiple *Alnus*-associated EM basidiomycete taxa also have the potential to associate with other hosts. Given the high frequency in which *Alnus* co-occurs with other EM host plants in western North America, additional studies in this region will be helpful in elucidating the processes involved in *Alnus* EM assemblage dynamics.

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REFERENCES

Avis PG, McLaughlin DJ, Dentinger BC, Reich PB, 2003. Long-term increase in nitrogen supply alters above- and below-ground ectomycorrhizal communities and increases the dominance of *Russula* spp. in a temperate forest savanna. *New Phytologist* **160**: 239–253.

Beccera A, Zak M, Horton T, Micolini J, 2005. Ectomycorrhizal and arbuscular mycorrhizal colonization of *Alnus acuminata* from Caliegua National Park (Argentina). *Mycorrhiza* **15**: 525–531.

Bormann B, Cromack K, Russell W, 1994. Influences of red alder on soils and long-term ecosystem productivity. In: Hibbs D, DeBell D, Tarrant R (eds), *The Biology and Management of Red Alder*. Oregon State University Press, Corvallis, pp. 47–56.

Brose U, Martinez N, Williams R, 2003. Estimating species richness: sensitivity to sample coverage and insensitivity to spatial patterns. *Ecology* **84**: 2364–2377.

Cajsa MR, Nygren R, Eberhardt U, Karlsson M, Parrent JL, Lindahl BD, Taylor AFS, 2008. Growth on nitrate and occurrence of nitrate reductase-encoding genes in a phylogenetically diverse range of ectomycorrhizal fungi. *New Phytologist* **180**: 875–889.

Chatarpaul L, Chakravarty P, Subramaniam P, 1989. Studies in tetrapartite symbioses I: role of ecto- and endomycorrhizal fungi and *Frankia* on the growth performance of *Alnus incana*. *Plant and Soil* **118**: 145–150.

Clarke K, Warwick R, 2001. *Change in Marine Communities: an Approach to Statistical Analysis and Interpretation*, 2nd edn. PRIMER V, Plymouth.

Cline E, Ammirati J, Edmonds R, 2005. Does proximity to mature trees influence ectomycorrhizal fungus communities of Douglas-fir seedlings? *New Phytologist* **166**: 993–1009.

Colwell R, 2005. *EstimateS: Statistical Estimation of Species Richness and Shared Species from Samples*. Version 7.5. User's guide and application published at: <http://purl.oclc.org/estimates> (accessed 15.06.09).

Colwell R, Coddington J, 1994. Estimating terrestrial biodiversity through extrapolation. *Philosophical Transactions of the Royal Society* **345**: 101–118.

Courty P, Pritsch K, Schloter M, Hartmann A, Garbaye J, 2005. Activity profiling of ectomycorrhiza communities in two forest soils using multiple enzymatic tests. *New Phytologist* **167**: 309–319.

DeBellis T, Kernaghan C, Bradley R, Widden P, 2006. Relationships between stand composition and ectomycorrhizal community structure in boreal mixed-wood forests. *Microbial Ecology* **52**: 114–126.

Erland S, Taylor A, 2002. Diversity of ectomycorrhizal fungal communities in relation to the abiotic environment. In: van der Heijden M, Sanders I (eds), *Mycorrhizal Ecology*. Springer, Berlin, pp. 163–200.

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

Geml J, Laursen G, Timling I, McFarland J, Booth M, Lennon N, Nusbaum C, Taylor D, 2009. Molecular phylogenetic biodiversity assessment of artic and boreal ectomycorrhizal *Lactarius* Pers. (Russulales: Basidiomycota) in Alaska, based on soil and sporocarp DNA. *Molecular Ecology* **18**: 2213–2227.

Guindon S, Gascuel O, 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52**: 696–704.

Hibbs D, DeBell D, Tarrant R, 1994. *Biology and Management of Red Alder*. Oregon State University, Corvallis.

Horton T, Bruns T, 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, Molina R, Hood K, 2005. Douglas-fir ectomycorrhizae in 40- and 400-year-old stands: mycobiont availability to late successional western hemlock. *Mycorrhiza* **15**: 393–403.

Ishida T, Nara K, Hogetsu T, 2007. Host effects on ectomycorrhizal fungal communities: insight from eight host species in mixed conifer-broadleaf forests. *New Phytologist* **174**: 430–440.

Jones M, Durall D, Harniman S, Classen D, Simard S, 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.

Kennedy P, Izzo A, Bruns T, 2003. There is high potential for the formation of common mycorrhizal networks between understorey and canopy trees in a mixed evergreen forest. *Journal of Ecology* **91**: 1071–1080.

Koide R, Xu B, Sharda J, Lekberg Y, Ositguy N, 2005. Evidence of species interactions within an ectomycorrhizal fungal community. *New Phytologist* **165**: 305–316.

Koljalg U, Dahlberg A, Taylor A, Larsson E, Hallenberg N, Stenlid J, Larsson K, Fransson P, Karen O, Jonsson L, 2000. Diversity and abundance of resupinate thelephoroid fungi as ectomycorrhizal symbionts in Swedish boreal forests. *Molecular Ecology* **9**: 1985–1996.

Koljalg U, Larsson K, Abarenkov K, Nilsson R, Alexander I, Eberhardt U, Erland S, Hoiland K, Kjoller R, Larsson E, Pennanen T, Sen R, Taylor A, Tedersoo L, Vralstad T, Ursing B, 2005. UNITE: a database providing web-based methods for the molecular identification of ectomycorrhizal fungi. *New Phytologist* **166**: 1063–1068.

Koo C, 1989. Water stress, fertilization, and light effects on the growth of nodulated mycorrhizal red alder seedlings. Ph.D. thesis, Oregon State University, Corvallis.

Kranabetter JM, Durall DM, MacKenzie WH, 2009. Diversity and species distribution of ectomycorrhizal fungi along productivity gradients of a southern boreal forest. *Mycorrhiza* **19**: 99–111.

Lilleskov EA, Hobbie EA, Fahey T, 2002a. Ectomycorrhizal fungal taxa differing in response to nitrogen deposition also differ in pure culture organic nitrogen use and natural abundance of nitrogen isotopes. *New Phytologist* **154**: 219–231.

Lilleskov EA, Fahey TJ, Horton TR, Lovett GM, 2002b. Belowground ectomycorrhizal fungal community change over a nitrogen deposition gradient in Alaska. *Ecology* **83**: 104–115.

Liu Y, Rogers S, Ammirati J, 1997. Phylogenetic relationships in Dermocybe and related *Cortinarius* taxa based on nuclear ribosomal DNA internal transcribed spacers. *Canadian Journal of Botany* **75**: 519–532.

Mejstrik V, Benecke U, 1969. The ectotrophic mycorrhizas of *Alnus viridis* (Chaix.) D.C. and their significance in respect to phosphorus uptake. *New Phytologist* **68**: 141–149.

Miller S, Koo C, Molina R, 1991. Characterization of red alder ectomycorrhizae: a preface to monitoring belowground ecological responses. *Canadian Journal of Botany* **69**: 516–531.

Miller S, Koo C, Molina R, 1992. Early colonization of red alder and Douglas fir by ectomycorrhizal fungi and *Frankia* in soils from the Oregon coast range. *Mycorrhiza* **2**: 53–61.

Molina R, 1979. Pure culture synthesis and host specificity of red alder mycorrhizae. *Canadian Journal of Botany* **57**: 1223–1228.

Molina R, 1981. Ectomycorrhizal specificity in the genus *Alnus*. *Canadian Journal of Botany* **59**: 325–334.

Molina R, Massicotte HB, Trappe JM, 1992. Specificity phenomena in mycorrhizal symbioses: community-ecological consequences and practical implications. In: Allen M (ed.), *Mycorrhizal Functioning*. Chapman and Hall, New York, pp. 357–423.

Molina R, Myrold D, Li C, 1994. Root symbioses of red alder: technological opportunities for enhanced regeneration and soil improvement. In: Hibbs D, DeBell D, Tarrant R (eds), *The Biology and Management of Red Alder*. Oregon State University, Corvallis, pp. 23–46.

Moreau P, Peintner U, Gardes M, 2006. Phylogeny of the ectomycorrhizal genus *Alnicola* (Basidiomycota, Cortinariaceae) based on rDNA sequences with special

emphasis on host specificity and morphological characters. *Molecular Phylogenetics and Evolution* **38**: 794–807.

Molina R, Trappe JM, 1982. Patterns of ectomycorrhizal host specificity and potential among Pacific Northwest conifers and fungi. *Forest Science* **28**: 423–458.

Navarro E, Bousquet J, Moiroud A, Munive A, Piou D, Normand P, 2003. Molecular phylogeny of *Alnus* (Betulaceae), inferred from nuclear ribosomal DNA ITS sequences. *Plant and Soil* **254**: 207–217.

Neal J, Trappe J, Lu K, Bollen W, 1968. Some ectotrophic mycorrhizae of *Alnus rubra*. In: Trappe J, Franklin J, Tarrant R, Hansen G (eds), *Biology of Alder*. USDA Forest Service, Portland, pp. 179–184.

Peay K, Bruns T, Kennedy P, Bergemann S, Garbelotto M, 2007. A strong species–area relationship for eukaryotic soil microbes: island size matters for ectomycorrhizal fungi. *Ecology Letters* **10**: 470–480.

Peay K, Kennedy P, Bruns T, 2008. Fungal community ecology: a hybrid beast with a molecular master. *BioScience* **58**: 799–810.

Peintner U, Bougher N, Castellano M, Moncalco J, Moser M, Trappe J, Vilgalys R, 2001. Multiple origins of sequestrate fungi related to *Cortinarius*. *American Journal of Botany* **88**: 2168–2179.

Peintner U, Moser M, Thomas K, Manimohan P, 2003. First records of ectomycorrhizal *Cortinarius* species (Agaricales, Basidiomycetes) from tropical India and their phylogenetic position based on rDNA ITS sequences. *Mycological Research* **107**: 485–494.

Peter M, Ayer F, Egli S, 2001. Nitrogen addition in a Norway spruce stand altered macromycete sporocarp production and below-ground ectomycorrhizal species composition. *New Phytologist* **149**: 311–325.

Posada D, Crandell K, 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**: 817–818.

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

Ronquist F, Huelsenbeck J, 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.

Seidl M, 2000. Phylogenetic relationships within *Cortinarius* subgenus myxacium, sections defibulati and myxacium. *Mycologia* **92**: 1091–1102.

Shainsky L, Radosevich S, 1992. Mechanisms of competition between Douglas-fir and red alder seedlings. *Ecology* **73**: 30–45.

Smith M, Douhan G, Kremier A, Rizzo D, 2009. Are true multihost fungi the exception or the rule? Dominant ectomycorrhizal fungi on *Pinus sabiniana* differ from those on co-occuring *Quercus* species. *New Phytologist* **182**: 295–299.

Tedersoo L, Jairus T, Horton B, Abarenkov K, Suvi T, Saar I, Koljalg U, 2008. Strong host preference of ectomycorrhizal fungi in a Tasmanian wet sclerophyll forest as revealed by DNA barcoding and taxon-specific primers. *New Phytologist* **180**: 479–490.

Tedersoo L, Suvi T, Jairus T, Ostonen I, Polme S, 2009. Revisiting ectomycorrhizal fungi of the genus *Alnus*: differential host specificity, diversity and determinants of the fungal community. *New Phytologist* **182**: 727–735.

Toljander JF, Eberhardt U, Toljander YK, Paul LR, Taylor AFS, 2006. Species composition of an ectomycorrhizal fungal community along a local nutrient gradient in a boreal forest. *New Phytologist* **170**: 873–884.

Trappe J, Fogel R, 1977. Ecosystematic functions of mycorrhizae. *Colorado State University Range Science Department Sciences Series* **26**: 205–214.

Yamanaka T, Li C, Bormann B, Okabe H, 2003. Tripartite association in an alder: effects of Frankia and *Alpova diplophloeus* on the growth, nitrogen fixation and mineral acquisition of *Alnus tenuifolia*. *Plant and Soil* **254**: 179–186.