Bacterial diversity associated with subalpine fir (*Abies lasiocarpa*) ectomycorrhizae following wildfire and salvage-logging in central British Columbia[1]

Madhukar B. Khetmalas, Keith N. Egger, Hugues B. Massicotte, Linda E. Tackaberry, and M. Jill Clapperton

Abstract: To assess the effect of fire and salvage logging on the diversity of mycorrhizal–bacterial communities, bacteria associated with *Cenococcum*, *Thelephora*, *Tomentella*, Russulaceae, and E-strain ectomycorrhizae (ECM) of *Abies lasiocarpa* seedlings were characterized using two approaches. First, bacteria were isolated and characterized by Biolog©, gas chromatography fatty acid methyl ester (GC-FAME), and amplified 16S rDNA restriction analysis (ARDRA). The bacterial communities retrieved from ECM from both sites were dominated by Proteobacteria (groups gamma and beta). *Pseudomonas* was the most common genus isolated, followed by *Variovorax*, *Burkholderia*, and *Xanthomonas*. Gram-positive isolates (mostly high-G+C Gram-positive bacteria) were more frequently retrieved on the burned–salvaged site, many commonly associated with the two ascomycete ECM, *Cenococcum* and E-strain. *Pseudomonas* species were retrieved more frequently from *Thelephora*. Although actinomycetes were isolated from all sites, almost no actinomycetes or other Gram-positive bacteria were isolated from either *Thelephora* or *Tomentella*. Second, amplified 16S rRNA gene sequences were amplified directly from root tips and then cloned into the plasmid vector pAMP1, followed by restriction analysis. This technique distinguished more genotypes than isolates retrieved by culturing methods, but generally, results were similar in that the largest proportion of the bacteria were putatively Gram-negative; putative Gram-positive bacteria were fewer and most were from the burned–salvaged site. Direct cloning resulted in many patterns that did not match any identified isolates, suggesting that a large proportion of clones were unique or not culturable by the methods used. Analysis for both protocols showed no significant difference in bacterial diversity between the burned–salvaged and unburned sites.

Key words: rhizosphere bacteria, ARDRA, 16S rDNA, Biolog©, GC-FAME.

Résumé : Afin d'évaluer l'impact d'un feu de forêt et d'une coupe de récupération sur la diversité des communautés de bactéries associées a des mycorhizes, nous avons caractérisé des bactéries associées avec des ectomycorhizes (ECM) de *Cenococcum*, *Thelephora*, *Tomentella*, des Russulacées et de la souche E de *Abies lasiocarpa* en ayant recours à deux approches. En premier lieu, des bactéries ont été isolées et caractérisées par Biolog©, par chromatographie en phase gazeuse des dérivés d'esters méthyliques d'acides gras (GC-FAME) et par analyse de restriction de l'ADNr amplifié (ARDRA). Les communautés bactériennes obtenues des ECM des deux sites étaient dominées par des protéobactéries (groupes gamma et bêta). Le genre isolé le plus fréquemment était *Pseudomonas*, suivi par *Variovorax*, *Burkholderia* et *Xanthomonas*. Les isolats Gram-positifs (principalement des bactéries Gram-positives à haut taux G+C) ont été plus fréquemment retrouvés sur le site brûlé et récupéré, plusieurs d'entre eux étant en association avec les deux ascomycètes ECM, *Cenococcum* et la souche E. Les espèces de *Pseudomonas* étaient plus fréquemment récupérées à partir de *Thelephora*. Bien que des actinomycètes ont été isolés à partir de tous les sites, pratiquement aucun actinomycète et aucune autre bactérie Gram-positive n'ont été isolés de *Thelephora* ou de *Tomentella*. En second lieu, les séquences du gène 16S de l'ARNr ont été amplifiées directement à partir des extrémités racinaires, puis clonées dans le vecteur plasmidique pAMP1, pour ensuite subir une analyse de restriction. Cette technique a permis de

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distinguer davantage de génotypes que des isolats récupérés par des méthodes de culture, mais les résultats étaient généralement semblables et ont permis de constater que la grande majorité des bactéries étaient potentiellement Gram-négatives; les bactéries Gram-positives étaient moins nombreuses, et la plupart provenaient du site brûlé et récupéré. Le clonage direct a produit plusieurs motifs qui ne correspondaient à aucun isolat identifié, ce qui indique qu'un pourcentage important des clones étaient uniques ou non cultivables par les méthodes utilisées. Une analyse effectuée à l'aide des deux protocoles n'a pas démontré de différence significative de la diversité bactérienne entre le site brûlé et récupéré et le site non brûlé.

Mots clés : bactéries de la rhizosphère, ARDRA, ADNr 16S, Biolog©, GC-FAME.

[Traduit par la Rédaction]

Introduction

Mycorrhizal fungi and their associated rhizosphere bacteria include a large and diverse group of microorganisms that mediate important plant and soil processes in boreal forest ecosystems. Ectomycorrhizal fungi have been shown to promote tree growth and establishment in forest ecosystems by facilitating nutrient and water availability (Dosskey et al. 1990; Bending and Read 1995*a*, 1995*b*; Smith and Read 1997) and by increasing protection against root pathogens (Schelkle and Peterson 1996). Soil bacteria include those that appear to enhance root colonization and ectomycorrhiza formation by specific fungi (mycorrhiza helper bacteria: Garbaye 1994) as well as those that promote growth of both ectomycorrhizal and nonmycorrhizal seedlings (plant growth promoting bacteria: Chanway and Holl 1992; Shishido et al. 1996*a*, 1996*b*). Root-associated bacteria may also be involved in nitrogen transformations. Li et al. (1992) and Li and Hung (1987) have shown that soil bacteria (including *Bacillus*, *Azospirillum*, and *Clostridium* species) isolated from Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) ectomycorrhizae (ECM) are capable of nitrogen fixation, possibly providing nitrogen to these plants.

Several studies have examined bacterial community structure associated with ECM. Axelrood et al. (1996) established a culture collection of 1820 bacterial strains from the roots of nursery- and forest-derived Douglas-fir seedlings that were putatively mycorrhizal. Bacteria were evaluated for their in vitro growth inhibition of plant fungal pathogens as well as human bacterial pathogens; inhibitory strains included *Streptomyces*, *Streptoverticillium*, *Bacillus*, *Pseudomonas*, and *Burkholderia* species. Massicotte et al. (1993) assessed the bacterial diversity of *Cenococcum* and *Rhizopogon* ECM of Douglas-fir in a 34-year-old plantation and a 125-year-old forest in southern British Columbia. In total, 58 taxa were described from these two morphotypes using standard culture techniques and Biolog© characterization; 15 bacterial taxa were identified as being unique to *Rhizopogon* and 24 to *Cenococcum* ECM. Frey et al. (1997) identified 300 isolates of fluorescent pseudomonads from Douglas-fir – *Laccaria bicolor* ECM as well as from the mycorrhizosphere and adjacent bulk soil. *Laccaria bicolor* appeared to exert a trehalose-mediated selection in favour of fluorescent pseudomonads in the vicinity of the ECM. Timonen et al. (1998) assessed bacteria from uncolonized soil, nonmycorrhizal and mycorrhizal short roots, as well as soil-colonizing external mycelium from intact *Pinus sylvestris* L. – *Suillus bovinus* (L. ex Fr.) O. Kuntze and *P. sylvestris* – *Paxillus involutus* (Batsch ex Fr.) Fr. mycorrhizospheres developed in microcosms. Fluorescent pseudomonads were commonly isolated from all mycorrhizosphere locations in nursery peat but were nearly absent from the forest humus community. Colony-forming units (CFU) for spore formers were generally higher in forest soils compared with nursery soil; however, Gram-negative bacteria were more common in both forest and nursery soil microcosms than Gram-positive/variable bacteria. To our knowledge, no studies have examined bacterial communities of subalpine fir (*Abies lasiocarpa* (Hook.) Nutt.) ECM.

The natural occurrence of fire in forest ecosystems and its use as a prescribed burning treatment in forest management generates many ecological questions. Studies that have examined the effect of fire on the soil microflora include Ahlgren and Ahlgren (1965), Jørgensen and Hodges (1970), and Bissett and Parkinson (1980). Population measurements have included estimates of fungal and bacterial biomass (Pietikäinen and Fritze 1993), cell counts or mycelial measures converted to biomass (Bissett and Parkinson 1980), as well as direct counts from fungal and bacterial cultures (Jørgensen and Hodges 1970). Some have examined the postfire microfungal community composition (Jørgensen and Hodges 1970; Bissett and Parkinson 1980), while others have assessed only ECM communities (Danielson 1984; Visser 1995; Baar et al. 1999; Jonsson et al. 1999; Mah et al. 2001). Few have explored postfire effects on bacterial–mycorrhizal associations. Fire has variably been reported to have no effect on bacterial and fungal populations, to decrease populations or, in several instances, to have a positive effect. Results have often been confounded by seasonal influences, such as timing and intensity of rainfall events, depth of sampling, intensity of burns, and the length of time since the fire (Ahlgren 1974; Bissett and Parkinson 1980).

Several studies have explored bacterial–mycorrhizal and (or) –rhizosphere associations using amplified rDNA restriction analysis and (or) DNA sequencing (Frey et al. 1997; Dunbar et al. 1999; Chow et al. 2002). Molecular techniques that sample bacterial DNA sequences directly from the environment generally show greater diversity compared with cultivation-dependent methods because many bacteria are not amenable to traditional isolation and culturing techniques (Frey et al. 1997; Dunbar et al. 1999).

The present study was established to examine the effect of wildfire plus salvage-logging on bacterial communities associated with ECM of naturally regenerating subalpine fir seedlings. It took place 4 and 5 years after a wildfire in the Sub-Boreal Spruce biogeoclimatic zone in the central interior of British Columbia. The focus of the study was to (*i*) characterize and compare bacteria associating with differ-

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ent subalpine fir ECM from an unburned and a burned plus salvage-logged site and (*ii*) compare rDNA amplicons from isolates obtained by standard culturing methods with rDNA amplicons obtained by direct DNA cloning techniques.

Materials and methods

Study sites

The study sites were located in the central interior of British Columbia (54°07′N, 122°04′W, elevation 600–750 m (Jull 1992)) within the Eagle fire area, a natural wildfire disturbance that burned over 2000 ha of forest in the summer of 1992. The area is in the Sub-Boreal Spruce biogeoclimatic zone, Willow wet cool variant (wk1) (Delong et al. 1996), and has a continental climate with a mean annual precipitation of 930 mm and temperatures averaging between –12 and 15°C (Jull 1992). Hybrid spruce and subalpine fir are the climax species (Meidinger et al. 1991). Soils are Brunisolic Gray Luvisols and Gray Luvisols formed on loam to clay glaciolacustrine deposits (DeLong et al. 1996); humus forms are mor and moder types (Meidinger et al. 1991).

Two sites were selected within the Eagle fire. One site was burned by the Eagle wildfire in 1992, subsequently salvage-logged over a period of 18 months (1992–1994), and replanted in the spring of 1995 with lodgepole pine (*Pinus contorta* Dougl. Ex Loud. var. *latifolia* Engelm.) and hybrid spruce (*Picea engelmannii* Parry ex Engelm. × *Picea glauca* (Moench) Voss) seedlings. Also growing on this site were naturally regenerating subalpine fir and hybrid spruce seedlings. An unburned stand (estimated at >100 years old) that was located adjacent to the burned site served as a control. It consisted of mature forest species including lodgepole pine, hybrid spruce, and subalpine fir, as well as young naturally regenerating hybrid spruce and subalpine fir.

Seedling sampling and ECM selection

Between October 8 and 21, 1996, subalpine fir seedlings (estimated at 3–4 years old) were sampled (seven each from the burned–salvaged site and the unburned site). Seedlings were harvested with soil surrounding the roots, bagged, and stored at 5°C until processed. Within 24–72 h, root systems were carefully washed free of soil, immersed in deionized water, and examined at low magnification using a stereomicroscope. Roots with ECM morphology were confirmed by preparing root squashes and viewing at high magnification using a compound microscope (Olympus CH-2, 100–1000×). Ectomycorrhizal morphotypes were characterized with reference to Agerer (1987–1998), Ingleby et al. (1990), Massicotte et al. (1994), and Goodman et al. (1996).

Three subalpine fir ECM morphotypes were selected from each of the two sites. When possible, the same morphotypes were chosen from the burned–salvaged and unburned sites; however, due to differences in morphotype occurrence and frequency on seedlings, selected types varied between sites. *Cenococcum* cf. *geophilum*, *Thelephora* cf. *terrestris*, and E-strain ECM were selected from seedlings on the burned–salvaged site; *C.* cf. *geophilum*, *Tomentella*, and a Russulaceae-like ECM were selected from the unburned site. *Cenococcum* was abundant in both sites, *Thelephora* and *Tomentella* were chosen because both are members of the Thelephoraceae, and E-strain and a Russulaceae type were chosen primarily because they occurred in sufficient numbers in burned–salvaged and unburned sites, respectively. For most comparisons, nine tips per morphotype (three replicate tips each from three seedlings) per site were selected. However, for the E-strain, we were able to sample only five tips from two seedlings, and 12 tips from four seedlings were harvested for *Cenococcum* on the burned–salvaged site. In total, 53 mycorrhizal tips from 14 seedlings were processed in 1996 for bacterial isolate analysis.

Between September 9 and 18, 1997, another 55 tips, representing the same morphotypes and following the same sampling procedures, were collected from 12 subalpine fir seedlings (five from the burned–salvaged site and seven from the unburned site). Due to its low frequency in 1997, all nine tips came from one seedling for *Cenococcum* on the burned–salvaged site. Cloning of the bacterial 16S rRNA gene was performed on these ECM without any attempt at culturing.

Isolation of bacteria from ECM

Ectomycorrhizal root tips up to approximately 5 mm long were washed 20 times by gently shaking in 1 mL each of sterile deionized water under aseptic conditions. An aliquot of 100 μL of the last wash was plated onto tryptic soy agar (TSA, 50%) (Difco, Detroit, Mich.) plates as a control to determine efficacy of washing. Each ECM tip was then transferred to a 1.5-mL microcentrifuge tube containing 100 μL of trypic soy broth (TSB, 50%), pH 7.0, and macerated with a micropestle (Mandel Scientific, Guelph, Ont.) under aseptic conditions. One hundred microlitres of serially diluted suspensions (10^{-1} and 10^{-2} in 50% TSB) were plated on TSA (50%) agar plates. Plates were incubated at 28°C for up to 7 days. One representative dilution plate was chosen for each tip, and 10 colonies were randomly selected using a numbered grid subdivided into 1-cm^2 sections. Colonies were purified by the streak plate method onto TSA (50%) and stored on TSA (100%) slants or cryopreserved at –80°C in TSB (100%) containing 20% glycerol.

Characterization of bacterial isolates

Of the 530 bacterial isolates retrieved, 231 were phenotypically characterized by the Biolog© identification system (Biolog Corp., Hayward, Calif.). Isolates were initially characterized by Gram staining, KOH treatment, and vancomycin resistance and then prepared and inoculated into GN or GP microplates (according to Biolog© specifications) and incubated at 28°C. Cultures that did not grow well at 28°C were incubated at 15–24°C for up to 48 h. Microplate test well reactions were analyzed using an automatic microplate reader (Biolog© Microstation) interfaced with an IBM compatible computer, and isolates were identified using the Biolog© Microlog database software (version 3.7) to species, genus, or closest match for Gram-negative and Gram-positive bacteria.

Of the original 530 isolates, 330 were sent to the Lethbridge Research Centre, Agriculture and Agri-Foods Canada, for gas chromatography fatty acid methyl ester (GC-FAME) analysis. These bacteria were grown on BBL trypticase soy broth agar (VWR Canlab, Mississauga, Ont.) at room temperature (20–23°C) for 3–5 days depending on growth rate. All cells on the plate were harvested, saponified, meth-

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ylated, and extracted for fatty acids according to standard procedures recommended for MIDI database comparison (MIDI, Newark, Del.). The extracts were analyzed using a gas chromatograph (Hewlett Packard 5898A) fitted with a Hewlett Packard Ultra 2 column using hydrogen as a carrier gas under the standard operating procedures recommended by MIDI. Bacterial isolates were identified using Sherlock software, which compares chromatographic fatty acid profiles with a database of both aerobic and anaerobic organisms (MIDI). Isolates that did not match the database were reported as either Gram-negative or Gram-positive based on the occurrence of odd-number and branched-chain fatty acids characteristic of Gram-positive bacteria or even-number, straight-chain, and cyclopropyl fatty acids characteristic of Gram-negative bacteria (Zelles et al. 1992).

Amplified 16S rDNA restriction analysis (ARDRA) of bacterial isolates

Of the original 530 isolates, 302 were successfully analyzed using 16S rDNA amplification and restriction endonuclease digestion. DNA extraction was done by heating 100 μL (approximately 3.8×10^8 CFU/mL sterile deionized water) at 95°C for 10 min (Hartung et al. 1993). The DNA extract was diluted 1:50 times and then subjected to polymerase chain reaction (PCR) amplification using 16S rDNA, eubacteria-specific 28-bp primers EubF (5′-ataggatccAACACATGCAAGTCGAACG) based on the primer Eub (Schmidt et al. 1991) and FGPSR (5′-atatctagaAGGAGGGGATCCAGCCGCA) based on the primer FGPS-1509-153 (Orso et al. 1994). The region in lowercase letters indicates restriction sites added to the primers to facilitate directional cloning. PCRs were performed using a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.) and 30-μL reaction mixtures that contained genomic DNA (approximately 32 ng), each of the four deoxynucleoside triphosphates (200 μM), primers (0.2 μM), 10× reaction buffer, $MgCl_2$ (2.0 mM), and *Taq* DNA polymerase (2.5 U) (Invitrogen, Burlington, Ont.). The reaction mixture was overlaid with a drop of sterile mineral oil (10 μL) (Sigma, St. Louis, Mo.) to reduce evaporation. A total of 35 cycles consisting of denaturation at 94°C for 45 s, annealing at 48°C for 45 s, and extension at 72°C for 2 min 18 s were carried out. A final extension of 72°C for 5 min was added to the last cycle. A reaction mixture with *Escherichia coli* genomic DNA and a sample without added DNA template acted as positive and negative controls, respectively. After 35 cycles, the PCR amplification products were checked by horizontal gel electrophoresis using a 0.7% agarose gel in 0.5× Tris–borate–EDTA (TBE) buffer (pH 8.0) (Sambrook et al. 1989). The molecular weight marker used was a 1-kb DNA ladder (Invitrogen). PCR-amplified products (approximately 1636 bp) were digested with 2 U of each of five restriction endonucleases, *Alu*I, *Cfo*I, *Hin*fI, *Mbo*I, and *Msp*I, at 37°C for 6 h. DNA fragments were separated on a 2.5% (1.5% NuSieve agarose and 1.0% agarose) gel containing ethidium bromide (10 μg/mL) with a 1-kb ladder marker, electrophoresed in 0.5× TBE buffer at 95 V for 3 h, and photographed under UV illumination with a Gel Print 2000i photodocumentation system.

Restriction fragment patterns generated for each isolate were analyzed using RFLPscan Plus, version 3.12 (Scanalytics, Fairfax, Va.). Amplified 16S rDNA restriction fragment databases generated for each seedling were compared at 6.0% match tolerance using RFLPscan Database, versions 2.1 and 3.12 (Scanalytics). Pairwise comparisons of all banding patterns were compiled for each database. Pairs of tips were matched using Dice's (1945) index (two times the number of common bands/two times the number of common bands plus the number of polymorphic bands), and then the index was converted to a distance value (i.e., 1 – Dice's index). Once all possible pairwise combinations were evaluated, a distance matrix was created and the Phi index (Mah et al. 2001) calculated.

Direct DNA analysis of bacteria from ECM

To assess bacterial community structure without cultural isolation, 16S rRNA gene sequences were amplified directly from 55 ECM tips, cloned into a plasmid vector, and then transformed colonies randomly sampled. To extract DNA from root tips, individual tips were washed (as described for isolation of bacteria) and then frozen at –80°C for 30 min in sterilized micro glass vials before grinding aseptically into a powder using a cold glass pestle (Mandel Scientific). The powdered root tissue was dispersed in 200 μL of 2× CTAB buffer (1.4 M NaCl, 100 mM Tris–HCl (pH 8.0), 20 mM EDTA (pH 8.0), 2% hexadecyltrimethylammonium bromide (CTAB), and 0.2% β-mercaptoethanol). The suspension was transferred to a microcentrifuge tube, the mortar and pestle were rinsed with another 200 μL of CTAB buffer, and the combined solution was placed in a 60°C water bath for 1 h. An equal volume of chloroform – isoamyl alcohol (24:1 v/v) was added and the tube mixed by inversion to form an emulsion. The emulsion was centrifuged for 10 min at 12 000 × *g* and the upper aqueous phase was transferred to a fresh 1.5-mL microcentrifuge tube. Nucleic acids were precipitated by adding an equal volume of cold isopropanol, inverting several times, and then incubating at –10°C for 5 min. Nucleic acids were pelleted by centrifugation at 12 000 × *g* for 10 min, and the isopropanol was removed by suction. The nucleic acid pellet was washed (twice) with 70% ethanol, centrifuged briefly, vacuum-dried overnight, and then resuspended in 25 μL of 8 mM NaOH and stored at –20°C until use.

Cloning of amplification products

Universal eubacterial primers Eub (5′-cuacuacuacuaAACACATGCAAGTCGAACG) and FGPS-1509-153 (5′-caucaucaucauAGGAGGGGATCCAGCCGCA) were synthesized with an additional 12-bp region (indicated in lowercase letters) that was utilized for directional cloning of amplification products. All PCRs were performed as above. Specific amplification of only bacterial 16S rDNA was confirmed using yeast and potato cell culture DNA as negative controls.

PCR-amplified products were separated on a preparative 0.7% low-melting-point agarose gel (NuSieve GTG, FMC) in 1× TAE (40 mM Tris–acetate, 2 mM EDTA) containing ethidium bromide, and full-length bands were then purified with a Wizard™ PCR prep purification system (Promega Corp., Madison, Wis.) and eluted with warm, sterile water. Recovered DNA was quantified with a GeneQuant II (Amersham Biosciences, Piscataway, N.J.) DNA quantification system before cloning. PCR primers were designed spe-

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cifically for use with the CloneAmp® pAMP1 system (Invitrogen/Canadian Life Technologies) for cloning PCR amplification products. Fifty nanograms of gel-purified 16S rDNA product was used per ligation. MAX efficiency DH5α™ competent cells (Invitrogen/Canadian Life Technologies) were transformed with 1 μL from each ligation. Transformants were screened by blue/white colony selection, and 10 white colonies were randomly selected from each root tip sample. Individual colonies were grown overnight in 1 mL of sterile LB (10 g of NaCl, 10 g of trypton, and 5 g of yeast extract in 1 L of deionized water, pH 7.0) containing 100 μg ampicillin/mL. One half of each overnight culture was cryopreserved at –80°C in 20% glycerol and the other half was frozen at –20°C for subsequent restriction fragment analysis.

Cloned inserts were reamplified directly from –20°C frozen bacterial cells utilizing the 20-bp primers pAMP1f (5′-CGCCTACTACTACTAAACAC) and pAMP1r (5′-CGA CATCATCATCATAGGAG). These primers span the junction of the plasmid cloning site and the PCR primer and were designed to avoid the coamplification of the *E. coli* DH5α 16S rRNA genes. PCRs were performed in 30-μL reaction mixtures (as above) and amplified similarly. After amplification, 5 μL of each reaction was analyzed on 0.7% agarose gels as above. Only those samples (472 clones) that contained a full-length insert (approximately 1636 bp) were analyzed by restriction enzyme digestion. Restriction fragments generated for each clone were compared using RFLPscan Plus at 6.0% match tolerance for comparisons across different gels. A fragment database composed of representatives of bacterial isolates identified by Biolog© and (or) GC-FAME, as well as cultures characterized as actinomycetes, was merged with databases of fragment patterns from cloned 16S rDNA sequences to identify putative Gram-negative and Gram-positive groups and to compare isolates obtained by the culture and direct amplification protocols (see Fig. 1).

Statistical analysis

Cultured bacterial isolates were compared at the genus level using the Biolog© and GC-FAME identities and standard diversity indices (richness, Shannon, Shannon evenness, and Simpson) (Magurran 1988). The number of genera and isolates per genus (Hill 1973; Ludwig and Reynolds 1988) were determined for each seedling by pooling all tips per seedling and keeping treatment (burned–salvaged and unburned) and morphotype (*Cenococcum*, the Thelephoraceae, and E-strain/Russulaceae) separate. Significant differences were determined by two-way analysis of variance (ANOVA) (JMP; SAS Institute Inc., Cary, N.C.) for treatment and morphotype ($p \leq 0.05$). Mean comparisons were tested for all morphotypes using the Tukey–Kramer HSD test ($p \leq 0.05$).

The Phi index (Mah et al. 2001) was used to assess treatment, morphotype, and method differences based on molecular samples of all isolates for which a 16S restriction pattern was obtained (i.e., identified and unidentified isolates, including actinomycetes). The Phi index uses pairwise distances (obtained from the Dice index distance matrices derived from restriction patterns) to calculate the index value instead of taxon-proportional abundance data on which traditional diversity indices are based. For a data matrix with $i = j$ rows and columns, the pairwise distances (d) for each sample were squared, summed, and then divided by $n - 1$ to give an average squared distance for each column, where n equals the total number of samples in the matrix. The Phi (Φ) index was calculated by summing the average squared distances of all of the columns divided by n:

$$\Phi = \frac{\sum_{j=1}^{n}\left[\frac{\sum_{i=1}^{n} d_{ij}^2}{n-1}\right]}{n}$$

where $i = j = n$.

The index ranges between 0, when all pairwise distances are equal to 0 (i.e., all fragment patterns are identical), and 1, when all pairwise distances are equal to 1 (i.e., no fragments are shared among any of the pairs of samples).

A three-way ANOVA of the Phi index data was used to test for significant differences between treatment (burned–salvaged versus unburned), method (culturing versus cloning directly from roots), and ECM morphotype. Since all nine tips for the cloned sample from *Cenococcum* on the burned–salvaged site came from one seedling, three pseudoreplicates were generated by randomly sorting the nine tips into groups of three, and the pseudoreplicates were used to make statistical comparisons.

Since the Phi index allows molecular databases for each seedling to be merged and then the Phi index recalculated from the pooled molecular patterns, additional tests were conducted to determine whether the Phi index calculated from pooled samples were significantly different from the Phi index calculated from their component samples. These tests were based on the assumption that if the component databases were sampling the same community, then the Phi index of the pooled samples should not be significantly different from the component samples; if the samples merged represent very different bacterial communities (i.e., containing divergent molecular banding patterns), then the Phi index calculated from the pooled samples would be expected to be greater than the Phi index calculated from the component samples. We tested these comparisons using a t test to determine if a single Phi index calculated from the pooled samples differed from the mean of the Phi indices calculated from the component samples (Sokal and Rohlf 1981).

Results

Taxonomic identification of bacterial isolates using Biolog© and GC-FAME

From the original 53 ECM root tips selected for culture analysis, 46 tips (representing all targetted seedlings and all morphotypes) produced isolates that were characterized by phenotypic methods and (or) amplified 16S rDNA restriction. No microbial growth occurred from water plated from the last ECM washes, suggesting that isolated bacteria were from within or closely associated with the ECM mantle and root tips. In general, many bacteria produced minute and

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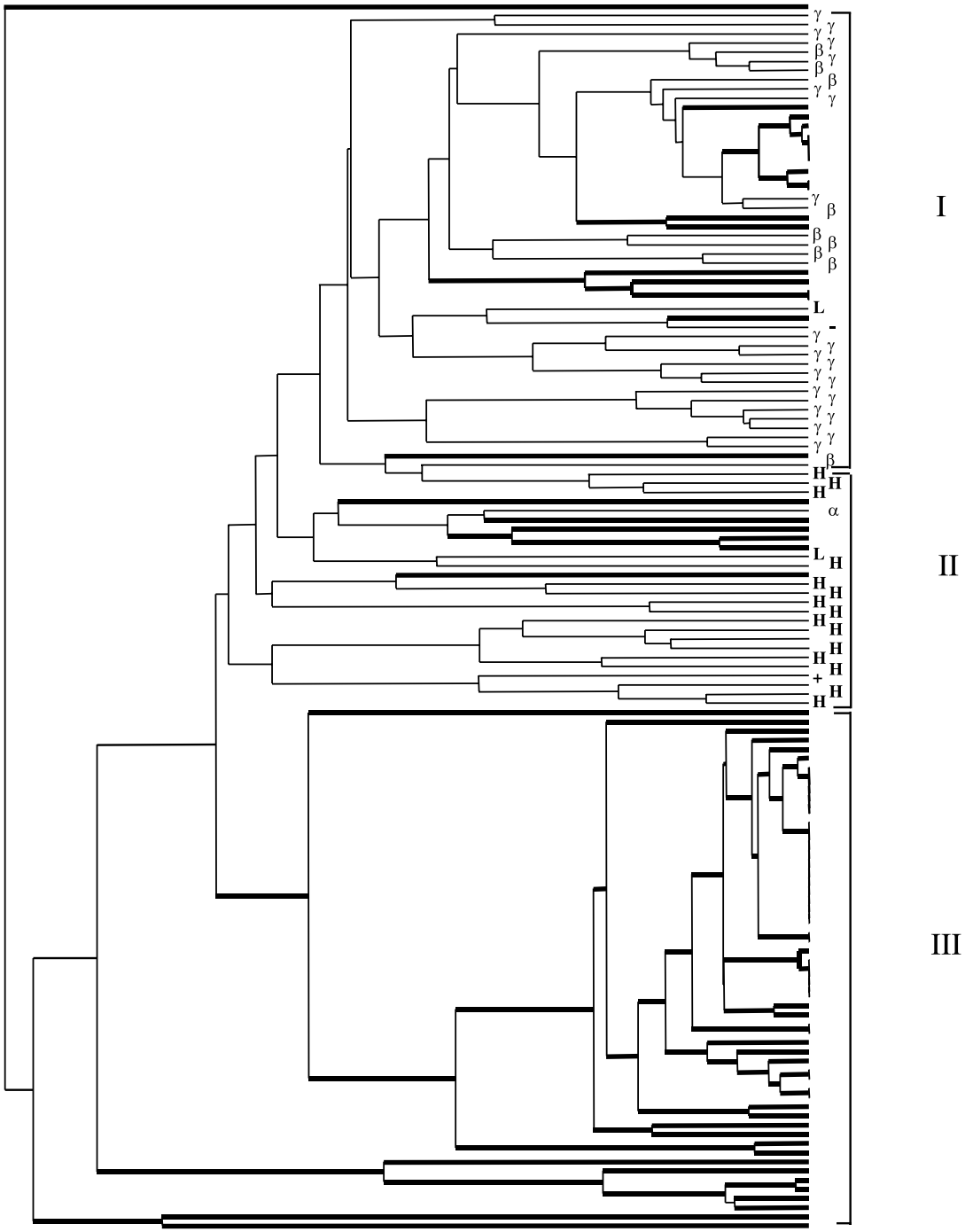
Fig. 1. Sample phenetic tree constructed by UPGMA analysis using the program NEIGHBOR in the software package PHYLIP (Felsenstein 1996) based on restriction patterns from amplified 16S rDNA (bold lines) obtained by direct cloning from *Cenococcum* ECM compared with amplified 16S rDNA restriction patterns from a sample of cultured isolates identified by Biolog© and (or) GC-FAME analysis, or colony morphology in the case of actinomycetes. Identified taxa have the following symbols: α, β, and γ, Proteobacteria; –, unclassified Gram-negative; L, low-G+C Gram-positive; H, high-G+C Gram-positive including actinomycetes; +, unclassified Gram-positive. Cluster I contains mostly Gram-negative isolates (Proteobacteria), although one member of the low-G+C Gram-positives clustered with this group. Cluster II contains mostly Gram-positive isolates (including isolates classified as actinomycetes based on colony morphology), although one member of the Proteobacteria clustered within this group. Cluster III contains clones that did not match any of the characterized bacterial samples from any of the identified groups and is hypothesized to contain isolates that could not be retrieved using traditional culture isolation methods.



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slow-growing microcolonies, yielding 4.2×10^1 to 2.5×10^4 CFU per root tip. Of the original 530 cultures, approximately 34% (180) lost their viability rapidly or could not be regrown after transfer from the original isolation plate. The remaining 350 were selected for characterization using Biolog©, GC-FAME, and ARDRA. Of these, 80 isolates did not regrow following subculturing for Biolog© characterization. Thirty-nine isolates were identified by colony morphology as actinomycetes; these were subjected to ARDRA but were not characterized by Biolog© or GC-FAME. Biolog© analysis of 231 isolates resulted in 167 identifications (72%); 64 (28%) did not match any entries in the Biolog© databases. Some of the unmatched Biolog© isolates and a few that did not regrow for Biolog© were identified by GC-FAME analysis or produced restriction fragment patterns. Biolog© similarity indices varied from 0.997 to 0.034; 102 (61%) had a similarity index ≥0.5, another 32 (19%) had a similarity index ≥0.4, and 21 (13%) had a similarity index ≥0.3 for a total of 93% of all Biolog© characterizations.

GC-FAME characterized 142 of 330 submitted isolates despite problems associated with retrieving and subculturing bacteria. GC-FAME similarity indices varied from 0.846 to 0.011; 33 (23%) had a similarity index ≥0.5, 25 (18%) had a similarity index ≥0.3, 72 (51%) produced an index between 0 and 0.2, and 12 (8%) did not match any isolates in the GC-FAME database.

Taxonomic identification was based on the level of the index value from the two characterization methods (Biolog© and GC-FAME) as well as on information obtained from the Gram stain, KOH, and vancomycin tests and from cell morphology (rod, coccus, filamentous). Characterized bacterial isolates and their similarity index values are listed in Table 1.

Treatment and ECM effects on culturable bacteria

Overall, 279 isolates, classified into 29 genera and representing 61 species and subspecies, were found associating with the five ECM morphotypes from the burned–salvaged and unburned sites (Table 1). The six most frequently isolated genera were *Pseudomonas* (28%), *Variovorax* (10%), *Burkholderia* (4%), *Xanthomonas* (3%), *Clavibacter* (3%), and *Corynebacterium* (3%). *Pseudomonas* isolates, although retrieved from all ECM and both treatments, were two to three times more frequent on *Thelephora* mycorrhizae from the burned–salvaged site compared with other ECM from both sites. This was due mostly to the abundance of *Pseudomonas fluorescens*. *Variovorax* isolates were six times more commonly isolated from the unburned site (*Cenococcum* and *Tomentella* mycorrhizae) (Table 1). The above six genera plus the actinomycetes represented over 65% of all species characterized.

With respect to treatment, twice the number of bacterial genera were retrieved from ECM from the burned–salvaged site compared with the unburned site (Table 2). In total, 55% (153 of all identified isolates) were characterized as Proteobacteria, most of these belonging to the groups gamma (35%) and beta (19%). Several were classified as alpha or "unclassified" bacteria. Gram-negative isolates were equally common to both treatments; however, those in the gamma group dominated the burned site whereas those in the beta group dominated the unburned site. Twenty-five percent of all isolates (70 strains) were characterized as Actinobacteria. Of these, 14% were classified as actinomycetes and occurred on both the burned and unburned sites; 11% were identified as other high-G+C Gram-positive bacteria and these were twice as frequent on the burned–salvaged site (Table 2). Four isolates were identified as low-G+C Gram-positive bacteria and one remained unclassified.

With respect to morphotype differences, approximately twice the number of genera and species were retrieved from *Cenococcum* from the burned–salvaged site compared with all other morphotypes, including *Cenococcum* from the unburned site (Table 1). In contrast with other morphotypes, almost no actinomycetes (one exception was characterized for *Tomentella*) and no *Corynebacterium* species were isolated from the Thelephoraceae ECM (*Thelephora* and *Tomentella*). In addition, only one Gram-positive taxon was identified from each of these two morphotypes (Table 1). No *Variovorax* or *Xanthomonas*, two genera that were present in low numbers on all other ECM, were isolated from either the E-strain (burned–salvaged) or the Russulaceae (unburned) ECM (Table 1). Despite these differences, diversity indices (richness, Shannon, Shannon evenness, and Simpson), based on the number of isolates per genus (Biolog© and GC-FAME data), showed no significant treatment, morphotype, or interaction effect ($p \leq 0.05$) (Table 3). Although not significant, diversity indices were greater for *Cenococcum* (both treatments) when compared with the Thelephoraceae, E-strain, and Russulaceae ECM (analysis not shown).

Molecular assessment of amplified 16S patterns from cultured and cloned samples

The 16S rRNA gene was successfully amplified from 302 of 530 bacterial cultures, each yielding the approximate 1636-bp PCR product corresponding to the expected product from the 16S rRNA gene. All amplified 16S rRNA genes were digested with each of the five restriction endonucleases, and restriction patterns were compared to generate the Phi diversity index.

With respect to cloning, 472 of the 540 clones retrieved were successfully amplified and digested with the five restriction endonucleases to generate restriction patterns. Amplification of 16S rDNA bacterial sequences from ectomycorrhizal root tip DNA and reamplification of cloned inserts yielded the expected 1636-bp PCR product. Restriction patterns from clones were compared with the database generated from the representative sample of ARDRA patterns from bacterial isolates identified by Biolog© and GC-FAME and from isolates identified by colony morphology as actinomycetes. Figure 1 shows an example of the combined analysis for clones associated with *Cenococcum* ECM (burned–salvaged site) and for cultures of characterized isolates compiled from all ECM morphotypes. It indicates that many clones did not match identified isolates.

A summary of results for bacteria following culture and clone assessments is presented in Table 4. Although we attempted to retrieve 10 colonies or 10 clones per root tip, analysis by direct bacterial cloning resulted in more clones compared with total numbers of isolates retrieved by standard culture techniques for all ECM in both treatments. Both treatments and protocols indicated a predominance of Proteobacteria. Overall, total numbers of Gram-positive iso-

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Table 1. Number of bacterial isolates identified (similarity index values in parentheses[a]) using Biolog© and GC-FAME analysis.

	Cenococcum		*Thelephora*	*Tomentella*	E-strain	Russulaceae
	Burned	Unburned	Burned	Unburned	Burned	Unburned
Gram-negative bacteria, Division: Gracilicutes						
Phylum: Proteobacteria						
Group alpha						
Agrobacterium tumefaciens A	3 (0.6–0.8)	0	0	0	0	0
Group beta						
Acidovorax avenae ssp. *avenae*	1 (0.5)	0	0	0	0	0
A. avenae ssp. *cattleyae*	0	3 (0.4)	0	0	0	0
A. delafieldii	0	0	0	1 (0.6)	0	0
Alcaligenes latus	2 (0.4, 0.8)	0	0	0	0	0
Burkholderia caryophylli	0	3 (0.4–0.6)	1 (0.5)	1 (0.5)	0	2 (0.5, 0.6)
B. solanacearum A	0	1 (0.6)	0	0	0	0
Burkholderia spp.	0	0	3[b] (<0.2)	0	0	0
Comamonas acidovorans	0	0	0	0	2 (0.4, 0.8)	0
Hydrogenophaga pseudoflava	2[b] (0.5)	0	0	0	0	0
Kingella denitrificans	0	0	0	0	1 (0.6)	0
Variovorax paradoxus	2 (0.5, 0.6)	9 (0.2–0.6)	2 (0.4, 0.6)	15 (0.5–0.7)	0	0
Variovorax spp.	0	0	0	1[b] (<0.2)	0	0
Group gamma						
Actinobacillus seminis	0	1 (0.2)	0	0	0	0
Chryseomonas luteola	0	0	2 (0.5, 0.7)	0	0	0
Flavimonas oryzihabitans	0	0	2 (0.3, 0.6)	0	0	0
Pantoea agglomerans	1[b] (<0.2)	1[b] (0.4)	0	0	0	0
Plesiomonas sp.	0	0	1[b] (0.2)	0	0	0
Pseudomonas andropogonis	1 (<0.2)	3 (0.5–0.6)	1 (0.6)	0	0	0
P. cichorii	0	0	1 (0.5)	0	0	0
P. coronafaciens	0	0	1[b] (0.8)	0	0	0
P. corrugata	1 (0.5)	0	4 (0.5–0.7)	0	0	0
P. floridana	1 (0.5)	0	0	0	0	0
P. fluorescens	1 (0.3)	0	13 (0.4–0.9)	0	0	2 (0.4, 0.6)
P. fuscovaginae	0	0	0	1 (0.6)	0	0
P. glathei	5 (0.2–0.9)	5 (0.4–0.7)	3 (0.6–0.7)	2 (0.4, 0.5)	8 (0.4–0.7)	3 (0.5–0.8)
P.-like group 2	0	0	0	0	1 (0.4)	0
P. phenazinium	0	0	0	0	1 (0.6)	0
P. putida	0	0	0	1[b] (0.4)	0	1 (0.8)
P. savastanoi	0	0	0	0	0	1 (0.6)
P. stutzeri	0	0	0	1 (0.4)	0	0
P. syringae	0	0	2[b] (0.4, 0.6)	0	0	0
P. tolaasii	1 (0.4)	1 (0.4)	3 (0.2–0.7)	0	0	2 (0.3, 0.6)
P. viridiflava	0	0	3 (0.3–0.6)	0	0	0
Pseudomonas spp.	1[b] (<0.2)	0	1[b] (0.2)	2[b] (<0.2)	0	0
Stenotrophomonas sp.	1[b] (0.2)	0	0	0	0	0
Xanthomonas oryzae pv. *oryzae*	1 (0.5)	1 (0.6)	0	0	0	0

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X. campestris pv. *aglaonema*	0	1 (0.6)	1 (0.6)	0	0	0
X. campestris pv. *hederae*	1 (0.2)	0	0	1 (0.5)	0	0
X. campestris pv. *vesicatoria*	0	0	0	4 (0.2–0.4)	0	0
Yersinia sp.	0	0	0	1[b] (<0.2)	0	0
Gram-negative (unclassified)						
Gilardi pink Gram-negative	1 (0.4)	0	0	0	0	4 (0.4–0.5)
Gram-positive bacteria, Division: Firmicutes						
Phylum: Actinobacteria (high-G+C group)						
Arthrobacter histidinolovorans	1 (0.9)	0	0	0	0	0
Cellulomonas fimi	0	0	0	0	0	1 (0.7)
C. turbata	3 (0.5–0.9)	1 (0.8)	0	0	0	0
Clavibacter michiganense	2[b] (0.7)	1[b] (0.5)	0	0	3[b] (0.4–0.7)	1[b] (0.8)
Corynebacterium ammoniagenes	1 (0.6)	0	0	0	0	0
C. jeikeium	1 (0.8)	2 (0.4, 0.6)	0	0	0	1 (0.7)
C. minutissimum	0	0	0	0	1 (0.4)	0
C. mycetoides	0	0	0	0	0	1 (0.5)
Curtobacterium flaccumfaciens	0	0	0	0	1[b] (0.7)	0
Microbacterium arborescens	0	0	0	0	3 (0.7–0.9)	0
Micrococcus luteus	0	0	0	0	1[b] (0.7)	0
M. varians	1 (0.5)	0	0	0	1[b] (0.7)	0
Rhodococcus erythropolis	0	0	0	1 (0.4)	0	0
R. fascians	2 (0.6, 0.7)	0	0	0	0	1 (0.9)
Actinomycetes[c]	8	5	0	1	13	12
Phylum: Endospora (low-G+C group)						
Bacillus gordonae	1 (0.4)	0	0	0	0	0
B. laterosporus	0	0	1[b] (0.4)	0	0	0
B. subtilis	1 (0.8)	0	0	0	0	0
Streptococcus faecium	1[b] (0.5)	0	0	0	0	0
Gram-positive (unclassified)						
CDC group E	1 (0.5)	0	0	0	0	0
Unidentified[d]	10	7	11	4	8	6
Total no. of isolates	59	45	56	37	44	38
Total no. of species	28	14	18	13	11	12
Total no. of genera	19	10	8	7	8	7
Sample size (no. of ECM root tips)[e]	11	8	8	7	5	7

Note: Bacteria were isolated from five ectomycorrhizal morphotypes of regenerating subalpine fir seedlings growing on burned–salvaged and unburned sites in the Eagle fire area. Classification follows Margulis and Schwartz (1998).

[a]Similarity index values are rounded to the closest decimal point. Values less than 0.2 are indicated by <0.2. The range is given for identities with more than two isolates.

[b]Identified only by GC-FAME (one exception, *Pseudomonas coronafaciens*, was also identified by Biolog© as a *Pseudomonas* sp.). All other identities are from Biolog©.

[c]Actinomycetes were classified based on filamentous growth form and were included in the ARDRA analysis but were not identified using Biolog© or GC-FAME.

[d]Isolates did not match any reference species in either the Biolog© or GC-FAME database.

[e]Forty-six ECM root tips (out of 53), representing all seedlings from all treatments, produced bacterial isolates.

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Table 2. Number of bacterial isolates and 16S rDNA clones recovered from subalpine fir mycorrhizae on burned–salvaged and unburned sites in the Eagle fire area.

	Treatment		
	Burned	Unburned	Both sites
Total Gram-negative	83 (91)	75 (158)	158 (249)
Total Gram-positive	26 (20)	10 (10)	36 (30)
Actinomycetes[a]	21	18	39
Unidentified[b]	29 (114)	17 (79)	46 (193)
No. of isolates	159 (225)	120 (247)	279 (472)
No. of species	47	30	61
No. of genera	27	13	29

Note: Bacteria were characterized using Biolog© and GC-FAME analysis (numbers in parentheses represent putative matches to main groups based on analysis of amplified 16S restriction patterns; see Fig. 1).

[a]Included in total number of isolates but not in total Gram-positive or number of species or genera.

[b]Isolates did not match any reference species in either the Biolog© or GC-FAME database.

lates and (or) clones were low for all morphotypes and both protocols, but in general, Gram-positive bacteria were more numerous from the burned–salvaged site. The number of unidentified isolates was greatest for *Cenococcum* and E-strain from the burned–salvaged site and Russulaceae from the unburned site; the percentage of unidentified bacteria was generally greater for clones than for cultures (Table 4).

Table 5 summarizes Phi index values by morphotype and treatment for both cloned and cultured samples and shows comparisons between treatments and protocols. The average Phi index value was higher for cultured *Cenococcum* bacterial isolates on the burned–salvaged site compared with the *Thelephora* and E-strain morphotypes, although the average Phi index using the cloning protocol was much more similar across morphotypes. Average Phi index values for cultured bacteria from the three morphotypes on the unburned site were more similar. Average Phi index values using the cloning protocol were always higher than the corresponding cultured sample for both treatments. Comparisons across treatments were inconclusive. Although the cultured *Cenococcum* community from the burned–salvaged site gave a much higher Phi index than on the unburned site, the cloning protocol suggested that the communities were similar in diversity. The *Thelephora* and E-strain bacterial communities on the burned site obtained by culturing gave lower average Phi index values than the corresponding *Tomentella* and *Russula* communities on the unburned site. However, the diversity of these communities obtained by cloning directly from ectomycorrhizal roots showed the opposite relationship.

The three-way ANOVA (treatment × protocol × morphotype) performed on the Phi index data revealed no significant differences by morphotype or treatment (burned–salvaged versus unburned), but the protocol (culturing versus cloning) was significant ($p < 0.01$). The interaction between treatment, protocol, and morphotype was also significant ($p < 0.05$).

In order to investigate how similar the ECM bacterial communities were on different seedlings, the molecular databases for each seedling were merged and the Phi index recalculated for the pooled sample (pooled Phi, Table 5). In most cases the Phi index was higher for the pooled sample than the average Phi index calculated from individual replicate samples, but there were no significant differences between average and pooled values for all morphotypes and both protocols at $p = 0.5$ based on a t test comparing the pooled value with the sample mean. The pooling of the *Thelephora* cultured samples was significant at $p = 0.1$. This suggests that the bacterial community was similar for each morphotype on each seedling, with the possible exception of the cultured *Thelephora* samples. It is interesting to note that the *Thelephora* and *Tomentella* communities were more comparable in diversity when the pooled Phi index values were compared.

When comparing the mean Phi index value with the pooled Phi values by treatment (merging burned–salvaged and unburned samples for each morphotype and protocol), the pooled Phi index values were generally slightly higher, although none were significantly greater based on a t test comparing the pooled value with the sample mean.

A comparison of the mean and the pooled Phi index values by protocol (merging cultured and cloned samples for each morphotype and treatment) showed the greatest differences in the Phi index values, with the pooled samples always higher and in many cases much higher than the mean Phi index value. Despite the greater differences, none of the individual pooled Phi index values were significantly greater than the average Phi value for each morphotype or treatment, although, as reported above, the ANOVA indicated that protocol overall was significant.

Discussion

Characterization of subalpine fir ECM-associated bacteria using Biolog©, GC-FAME analysis, ARDRA of cultured isolates, and rDNA cloned directly from roots showed no significant difference in bacterial diversity between burned–salvaged and unburned sites, suggesting that bacterial diversity was unaffected by disturbance 4–5 years after fire and salvage-logging. However, both culture and direct cloning protocols did suggest that Gram-positive diversity was higher in the burned–salvaged site than in the unburned site and that Proteobacteria groups beta and gamma dominated both treatment sites.

In a study on bacterial community structure, Timonen et al. (1998) found that Gram-negative bacteria predominated in both forest- (62%) and nursery-derived (86%) Finnish soils. Garbaye and Bowen (1989) isolated culturable bacteria from within and on the mantles of *Rhizopogon*–pine ECM grown in field-derived soil and calculated that 75% were Gram-negative, many being fluorescent pseudomonads. Spore formers and actinomycetes were less numerous. Timonen et al. (1998) found fewer Gram-positive/variable bacteria from forest humus soil than from nursery peat as well as an increased dominance of *Bacillus* species in the outer mycorrhizosphere and uncolonized soil. Massicotte et al. (1993) retrieved numerous Gram-positive bacteria (mainly bacilli) from both *Cenococcum*– and *Rhizopogon*–Douglas-fir mycorrhizae. The majority of Gram-positive isolates identified in our study were high-G+C Gram-positive strains and actinomycetes; *Bacillus* species accounted for

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Table 3. ANOVA for diversity indices (richness, Shannon, Shannon eveness, and Simpson) showing treatment (burned–salvaged and unburned) and morphotype effect for bacteria associating with subalpine fir mycorrhizae on the Eagle fire site.

	Treatment effect		Morphotype[a] effect		Treatment × morphotype	
	F	*p*[b]	*F*	*p*	*F*	*p*
Richness	0.6434	0.4381	3.2284	0.0755	0.2581	0.7767
Shannon	0.1953	0.6664	2.2979	0.1429	0.3506	0.7112
Evenness	0.0038	0.9517	1.1701	0.3434	0.2846	0.7573
Simpson	0.0018	0.9670	1.6161	0.2391	0.3208	0.7316

Note: Indices were based on the number of identified cultured isolates retrieved per genus using Biolog© and GC-FAME data.

[a]Morphotypes included *Cenococcum*, two Thelephoraceae (*Thelephora* and *Tomentella*), E-strain, and Russulaceae.

[b]Mean comparisons for morphotypes were tested using the Tukey–Kramer HSD test; p values ≤ 0.05 were considered to be significant.

Table 4. Comparison between genotypes (ARDRA) and isolates (Biolog© and GC-FAME analysis) of bacteria associated with five ectomycorrhizal morphotypes of regenerating subalpine fir growing on burned–salvaged and unburned sites in the Eagle fire area.

	Cenococcum				*Thelephora*		*Tomentella*		E-strain		Russulaceae	
	Burned		Unburned		Burned		Unburned		Burned		Unburned	
	Clone	Culture	Clone	Culture	Clone	Culture	Clone	Culture	Clone	Culture	Clone	Culture
Gram-negative	26.8	44.1	66.7	64.4	53.7	78.6	91.3	83.8	43.4	29.5	34.9	39.5
Gram-positive	2.4	25.4	7.1	8.9	19.4	1.8	1.3	2.7	6.6	22.7	3.6	13.2
Unidentified[a]	70.7	16.9	26.2	15.6	26.9	19.6	7.5	10.8	50.0	18.2	61.5	15.8
Actinomycetes[b]	—	13.5	—	11.1	—	0	—	2.7	—	29.5	—	31.6
Total (*n*)	82	59	84	45	67	56	80	37	76	44	83	38

Note: Values presented are percentages except for totals (*n*), which are actual values (isolates and clones).

[a]Isolates did not match any of those in the Biolog© or GC-FAME databases; clones did not match any patterns in the amplified 16S rDNA restriction fragment databases.

[b]Included in total number of isolates but not in total for Gram-positive bacteria (cultures).

only three strains. It is possible that some bacilli were not retrieved by the culture methods used. Pasteurizing soil samples (Timonen et al. 1998), or heat treatment of soil suspensions, or amending TSA with the antibiotic nalidixic acid (Axelrood et al. 2002*a*) might improve the ability to retrieve Gram-positive cultivable species.

Direct 16S rDNA bacterial cloning produced many clones that did not match amplified 16S rDNA restriction patterns of any of the characterized cultured isolates. Of particular interest was the large group of clones that clustered separately from the identified cultured isolates (represented by Proteobacteria groups alpha, beta, and gamma, the high-G+C Gram-positive bacteria, including actinomycetes, and the low-G+C Gram-positive bacteria). This cloned group (Cluster III in Fig. 1) was clearly differentiated from the cultured isolates, despite the nonoptimized UPGMA analysis and the relatively low information content of restriction fragment data. Without DNA sequencing, we cannot determine the phylogenetic affinities of this group, but they are clearly distinct from those of the identified cultured isolates. Of the clones that could be assigned to putative Gram-negative and Gram-positive groups, most were Gram-negative.

In a study using phenotypic methods in parallel with 16S rRNA cloning analyses for soil DNA, Dunbar et al. (1999) assessed bacterial community diversity of two pinyon pine rhizosphere soils and "between-tree" soils. The two methods resulted in different measures of richness for one of the four environments but identified similar levels for evenness; both methods indicated one environment to be distinct from the other three. In contrast with our study, low-G+C Gram-positive bacteria (mostly bacilli) dominated their culture collection. Differences between their study and ours, in retrieval of Gram-positive versus Gram-negative bacteria, may be directly related to the fact that they targeted the rhizosphere, not ECM, as in the present study.

Cultivation-dependent (Axelrood et al. 2002*a*) and cultivation-independent (Axelrood et al. 2002*b*) methods have been used to assess forest soil bacterial diversity with respect to soil type, soil disturbance, and season. Bacterial isolates and 16S clones were classified into three and nine bacterial divisions, respectively. GC-FAME analysis of bacterial isolates and 16S rRNA gene analysis of 16S-clones indicated that 25% of isolates and 13% of 16S clones could not be conclusively classified to a bacterial division. Soil disturbance treatments (soil compaction and organic matter removal) influenced the recovery of Actinobacteria isolates and the member genus *Arthrobacter* and gamma Proteobacteria 16S clones and the member genus *Pseudomonas* from mineral soil samples. As in the present study, Proteobacteria 16S clones were well represented in 16S clone libraries from forest soil (Axelrood et al. 2002*b*) and lodgepole pine rhizosphere soil (Chow et al. 2002).

Higher estimates of bacterial diversity when using cloning techniques versus culturing are presumed to be due to cloning of bacteria that are not amenable to traditional phenotypic methods and identification (including cultured

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Table 5. Phi index values based on amplified 16S restriction patterns for identified and unidentified bacterial isolates (cultured) and PCR products reamplified from clones obtained directly from root tips (cloned) of five ectomycorrhizal morphotypes of naturally regenerating *Abies lasiocarpa* seedlings growing on burned–salvaged and unburned sites in the Eagle fire area.

Morphotype	*n*	Mean Phi (SE)	Pooled Phi	*t* statistic[a]
Burned–salvaged site				
Cenococcum (cultured)	4	0.299 (0.044)	0.333	0.348
Cenococcum (cloned)	3	0.305 (0.060)	0.331	0.219
Thelephora (cultured)	3	0.087 (0.014)	0.198	3.972
Thelephora (cloned)	3	0.380 (0.048)	0.387	0.077
E-strain (cultured)	2	0.094 (0.052)	0.104	0.118
E-strain (cloned)	3	0.327 (0.041)	0.341	0.172
Unburned site				
Cenococcum (cultured)	3	0.122 (0.021)	0.125	0.078
Cenococcum (cloned)	3	0.312 (0.015)	0.316	0.125
Tomentella (cultured)	3	0.136 (0.036)	0.157	0.297
Tomentella (cloned)	3	0.292 (0.019)	0.311	0.518
Russula (cultured)	3	0.197 (0.092)	0.274	0.421
Russula (cloned)	3	0.259 (0.004)	0.256	–0.302
Treatments merged (burned–salvaged + unburned)				
Cenococcum (cultured)	7	0.223 (0.044)	0.256	0.269
Cenococcum (cloned)	6	0.309 (0.028)	0.346	0.510
Thelephora × *Tomentella* (cultured)	6	0.112 (0.020)	0.199	1.627
Thelephora × *Tomentella* (cloned)	6	0.336 (0.030)	0.350	0.179
E-strain × *Russula* (cultured)	5	0.155 (0.059)	0.206	0.352
E-strain × *Russula* (cloned)	6	0.293 (0.024)	0.309	0.256
Protocols merged (cultured + cloned)				
Cenococcum (burned)	7	0.301 (0.033)	0.407	1.141
Cenococcum (unburned)	6	0.217 (0.044)	0.277	0.513
Thelephora (burned)	6	0.234 (0.069)	0.338	0.572
Tomentella (unburned)	6	0.214 (0.039)	0.299	0.821
E-strain (burned)	5	0.234 (0.064)	0.290	0.362
Russula (unburned)	6	0.228 (0.043)	0.340	0.978

Note: Sample size (*n* = number of seedlings), mean Phi index value, standard error of the mean (in parentheses), pooled Phi index value, and *t* statistic of the comparison between the mean and the pooled Phi index value are presented.

[a]No *t* statistics were significant at $p = 0.05$ (see text for discussion).

isolates that are lost or unable to regrow after subculturing). Gilbert et al. (1993) described similar problems maintaining field-derived bacteria. Working with soil bacteria involves challenges with respect to isolate and clone characterization, and Dunbar et al. (1999) cautioned that biases exist with both plating and 16S rDNA cloning that can lead to distortions in diversity assessments. Bacteria present in low numbers may be missed, database libraries may lack isolates or clones, and environmental factors such as drought may favour or disfavour surviving organisms (Stackebrandt et al. 1993). To improve on our analyses of isolates and clones, 16S rDNA sequencing and phylogenetic identification could provide a major advantage.

We did not detect significant differences in diversity between burned and unburned treatments. Reducing the number of morphotypes and increasing the sample size for each ECM type might improve assessment when diversity is very high. The number of targeted root tips (three tips per seedling, total of nine) was slightly lower than in Timonen et al. (1998) who assessed five short roots per sample (total of 20) or three dichotomous tips (total of 12) and in Frey et al. (1997) who selected 10 ECM root tips per seedling (total of 40). These authors appeared to have pooled their ECM tips into one sample (per seedling) whereas all ECM tips were processed individually in the present study. Selecting 10 random isolates or clones per root tip was similar to the strategy of Timonen et al. (1998) who collected 10 colonies from plates containing the general medium TSA.

It is also possible that some resolution of differences between cultured and cloned bacteria isolates was lost due to sampling over 2 years (fall 1996 and 1997). Sampling each seedling concurrently for both culturable and cloned bacteria would reduce this factor. However, a study by Shaffer et al. (2000) did sample litter and soil from Douglas-fir forests and adjacent clearcuts at three time intervals (May, July, and September) over a 16-month period (1996–1997). They found high consistency in the community structure of diazotrophs, as assessed by *nifH* gene amplification. The characteristics of the clearcut (cut 1988, burned 1990, and replanted 1993) were comparable with those of our sites, which suggests that we should not anticipate significant deviations in the community structure of our sites over the 11-month period between our samples.

The Phi index was used to compare the restriction fragment patterns because traditional indices, based on species proportional abundance, are difficult to apply to molecular

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patterns. This index is expected to be less affected by intraspecific variation, since unique genotypes with small pairwise distance values contribute relatively little to the average squared genetic distance. Conversely, unique genotypes with large pairwise distance values contribute more to the average squared genetic distance, which would make the index more sensitive to diverse bacterial genotypes.

An advantage of the Phi index is that samples can be merged to test hypotheses about their genetic similarity. When samples are merged, large deviations from expected Phi values suggest that the samples represent bacterial communities that contain different components of phylogenetic diversity. When replicate seedling samples were combined in this study, mean and pooled Phi index values remained similar. However, when treatment (burned–salvaged and unburned) samples were merged, the pooled index tended to be higher (although not significant), suggesting that the bacterial community composition differed between treatments. Mean Phi index values were also lower for amplified 16S patterns (all isolates) from cultured bacteria (0.087–0.299) than those from clones (0.259–0.380), suggesting that phylogenetic diversity of the cultured samples was less than that of the cloned samples. When protocol (cultured and cloned) databases were merged, the pooled Phi index values were always higher, indicating that cloning most likely sampled large numbers of bacteria that were not amenable to isolation or to stable growth on culture media tested in this study. This observation is consistent with the UPGMA cluster analysis (see Fig. 1).

Overall, the Biolog© characterization success was low but similar to other reports in the literature for culturable bacteria (Miller and Rhoden 1991; Timonen et al. 1998). Historically, Biolog© and GC-FAME databases have consisted of mostly relatively fast-growing heterotrophic bacteria that are easily cultured whereas many soil and environmental bacteria are often slow-growing and may have less affinity to routine culture substrates (Miller and Rhoden 1991). Nevertheless, Biolog© and GC-FAME databases are improving continuously and increased confirmation of identifications of culturable bacteria and clones by molecular techniques will enable a better comparison of protocols in future studies.

Although we were unable to identify clear fungal-specific selection with respect to bacterial communities, some observations and trends are of interest. More species and genera were retrieved from *Cenococcum* from the burned–salvaged site than from the other morphotypes, and cloning resulted in the highest occurrence of amplified 16S rDNA restriction patterns that did not match identified cultured isolates. In total, 34 bacterial species were identified by culturing for *Cenococcum* compared with 27 for *Thelephora* and *Tomentella* and 20 for the E-strain and Russulaceae. Massicotte et al. (1993) isolated 43 taxa associating with *Cenococcum* – Douglas-fir ECM in southern British Columbia, many of which were Gram-positive.

Pseudomonas species were most commonly retrieved from *Thelephora* from the burned–salvaged site compared with all other morphotypes on both sites. *Thelephora* is known to form ECM with many host species, including planted seedlings on disturbed sites and greenhouse seedlings (Danielson 1991; Massicotte et al. 1998, 1999) as well as naturally regenerating seedlings from clearcuts (Mah et al. 2001) and from wildfire and salvage-logging sites (Egger and Massicotte 2000). It is less common on seedlings in mature forest sites. *Pseudomonas* species also colonize ECM seedlings from nurseries and greenhouses (Garbaye and Bowen 1989; Garbaye 1994) as well as field ECM (Massicotte et al. 1993) and rhizosphere soils (Timonen et al. 1998). Isolates of *P. fluorescens* can act as mycorrhiza helper bacteria and may selectively promote the establishment of mycorrhizal symbiosis (Garbaye 1994; Frey-Klett et al. 1997). Garbaye and Duponnois (1992) and Garbaye (1994) described *Thelephora* ECM and fungal cultures that were challenged with two *Bacillus* and two *Pseudomonas* (one *P. fluorescens*) strains originating from *Laccaria laccata* – Douglas-fir ECM and *L. laccata* sporocarps. They found a negative effect, reduced ECM formation, and mycelial growth, for *Thelephora* as well as *Cenococcum* and suggested that some bacteria may be fungus specific but not plant specific. The relationship between *Thelephora* and its associated bacteria and the concept of plant–fungal–bacteria relationships could be explored further.

Although actinomycetes form a substantial part of the microbial flora in forest ecosystems (Rozycki 1987; Strzelczyk and Szpotanski 1989; Axelrood et al. 1996) and were commonly retrieved in our study, neither the *Thelephora* nor the *Tomentella* ECM had many actinomycetes or Gram-positive bacteria that could be cultured. Some *Streptomyces* produce antibiotics and secondary metabolites that have antifungal properties (Axelrood et al. 1996), and under laboratory conditions, actinomycetes have both stimulated and inhibited growth of ectomycorrhizal fungi (Richter et al. 1989). Richter et al. (1989) isolated actinomycetes from ECM roots and then tested them against *Laccaria* and *Thelephora* ECM fungi. Most *Thelephora* cultures were negatively affected by the actinomycetes, which is consistent with the low numbers isolated in this study.

E-strain ECM are predominantly formed by fungi in the genus *Wilcoxina* (Egger 1996). Russulaceae ECM are formed by basidiomycetes and are commonly found in mature forest sites (Kernaghan et al. 1997; Visser et al. 1998). Twice the number of Gram-positive isolates were retrieved from E-strain ECM compared with Russulaceae ECM whereas numbers of Gram-negative bacteria and actinomycetes were similar between the two morphotypes and sites. Cloning resulted in many unidentified bacteria for both morphotypes. Interestingly, E-strain and *Cenococcum*, both ascomycetes, were frequently identified on seedlings on the burned–salvaged site; it is possible that these fungi may also be selectively associating with Gram-positive bacteria occurring on burned or disturbed sites.

In summary, the study results suggest that the ECM bacterial community 4–5 years after wildfire and salvage-logging was similar to that on the unburned site, although high-G+C Gram-positive bacterial diversity was elevated in the burned treatment. There was a lack of clear identification of ECM-specific selection within bacterial communities, but several interesting trends were observed. Increasing the sample size of culturable isolates and improving identification protocols, perhaps through the use of sequencing techniques, might facilitate these assessments. The study identified differences between culturable and nonculturable ECM bacterial communities, generally supporting and extending much of the

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recent theory in microbial ecology. It adds to a rather limited body of information on ECM bacterial associations that will no doubt expand with advances in techniques over the coming years.

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