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Article *in* Boreal Environment Research · January 2006

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BOREAL ENVIRONMENT RESEARCH 11: 45–53 ISSN 1239-6095
Helsinki 20 February 2006

Actinobacterial communities of boreal forest soil and lake water are rich in mycobacteria

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Received 29 Mar. 2005, accepted 23 June 2005 (Editor in charge of this article: Raija Laiho)

Niva, M., Hernesmaa, A., Haahtela, K., Salkinoja-Salonen, M., Sivonen, K. & Haukka, K. 2006: Actinobacterial communities of boreal forest soil and lake water are rich in mycobacteria. *Boreal Env. Res.* 11: 45–53.

The actinobacterial communities present in two Finnish lakes and in the surrounding conifer forest soil were investigated using DNA based methods. The dominant actinobacteria in the soil were found to belong to genus *Mycobacterium*. Therefore specific primers were designed and tested to study the mycobacterial communities in boreal environment more closely. The denaturing gradient gel electrophoresis (DGGE) and sequencing analysis showed that the microbial populations in lakes were different from those in the surrounding soil. Thus, each of the environments had their own actinobacterial and mycobacterial populations. The majority of the obtained mycobacterial sequences were closely related to the described species of environmental mycobacteria, some of which are pathogenic. However, several sequences were not closely related to any presently known species. The chemical characteristics of boreal forest soil seem to favour proliferation of mycobacteria, many of which still remain to be isolated.

Introduction

Actinobacteria, former actinomycetes, form a major bacterial population in soil. As versatile aerobic chemo-organotrophic gram-positive bacteria they have been recovered from a wide variety of environmental sources, where they can act as saprophytes, symbionts, parasites or even pathogens (Williams *et al.* 1983). Together with the fungi, actinobacteria are the most important producers of exocellular enzymes (Paul and Clark 1996) vital for nutrient recycling in soil. Most actinobacteria favour neutral pH but genus *Mycobacterium* colonises also acid soils. Mycobacteria are able to utilise a large variety of hydrocarbons including branched-chain, unsaturated, aromatic and cyclic hydrocarbons that are present in different forms in pristine boreal environment and in contaminated sites (Krulwich and Pellicione 1979, Hartmans and Bont 1992, Coleman *et al.* 2002). In fact, mycobacteria have been isolated from acid soils and waters more frequently than from environments of neutral pH (Brooks *et al.* 1984, Iivanainen *et al.* 1993, 1999a, 1999b, Iivanainen 1995, Kirschner *et al.* 1999). These mycobacteria are usually referred to as environmental or non-tuberculous mycobacteria. The

number of human non-tuberculous infections is increasing (Kirschner *et al*. 1992, Katila *et al*. 1995, Falkinham 1996, 1999, Wallace *et al*. 1998, Primm *et al*. 2004). As these do not transmit from person to person their source is believed to be environmental, but the reservoirs are not yet well understood.

We studied the actinobacterial and mycobacterial communities in a boreal forest soil and water ecosystem typical of Finland. The typical Finnish forest ecosystem is characterized by acidic and nutrient poor podzolic soils and small lakes with high content of humic matter (Larsen 1980). We asked the question whether the lakes that receive runoff from the surrounding forests have a community structure similar to that of the forest soil. Many actinobacteria are known to be slow-growing, months are required to get a colony on an agar plate, and many remain uncultivable. Therefore molecular methods in studying the bacterial communities were chosen (Heuer *et al*. 1997, Ross *et al*. 1997, Schwartz *et al*. 1998, Covert *et al*. 1999, Cheung and Kinkle 2001). We used actinobacteria- and mycobacteria-specific PCR primers to amplify partial 16S rRNA genes from the DNA extracted directly from soil and water. The PCR products were analyzed by denaturing gradient gel electrophoresis (DGGE), which resolves DNA fragments of similar length but different nucleotide sequence, and by sequencing.

Materials and methods

Sampling

The study site was located in Nastola, southern Finland (61°00´N, 25°50´E) and was nutrient poor mixed forest dominated by Scots pine (*Pinus sylvestris*) with ground vegetation of lingonberry (*Vaccinium vitis-idaea*) and mosses (mainly *Dicranum* spp. and *Pleurozium* spp.). The podsolized soil was sampled twice (Oct. 1997 and Oct. 1998) with a Westman corer (Westman 1995) from the forest slope leading to Lake Sammalisto (described below) nearby. Three soil core replicates were collected and divided into three layers according to the color of the layer. The soil layers were the vegetation layer including litter layer and all small vegetation e.g. moss (thickness 5–10 cm, average pH 5.0, dry matter 20%, organic matter 93% and total nitrogen 16 mg g^{-1} dw), the humus layer (thickness 4–8 cm, average pH 4.4, dry matter 33%, organic matter 75% and total nitrogen 12 mg g^{-1} dw) and the mineral soil including the eluvial and illuvial horizons (10 cm or less, average pH 5.1, dry matter 78%, organic matter 8% and total nitrogen 1 mg g^{-1} dw). The samples were sieved and stored at –20 °C.

In the area ca. 40 small lakes were situated in the headwater area. Since the area was rocky, watersheds around the lakes were small with distinct boundaries. Water was sampled from Lake Ahvenlammi, a meso-oligotrophic clear-water lake (water colour 10 mg Pt l^{-1}, average pH 5.8, total nitrogen 387 μg l^{-1} and total phosphorus 14 μg l^{-1}, max depth 12 m, area 0.12 km^2 and catchment area 0.44 km^2) and from Lake Sammalisto, a meso-oligotrophic humic lake (water colour 40 mg Pt l^{-1}, average pH 5.7, total nitrogen 477 μg l^{-1} and total phosphorus 14 μg l^{-1}, max depth 7 m, area 0.12 km^2 and catchment area 2.20 km^2), further details were published by Haukka *et al*. (2005). The lakes were sampled at the time of thermal stratification (Aug. 1997 and Sep. 1998) from epilimnion, thermocline and hypolimnion and during the autumn turnover (Oct. 1997 and Oct. 1998). The samples were lifted with a Limnos sampler at one-meter vertical intervals. The waters within each stratum were combined and used to fill up 10-litre containers, stored at +4 °C over night and then concentrated with Pellicon tangential filtration system (Watson-Marlow 601S) using 1 bar pressure and membrane of pore size 0.16 μm. Retentate, approx. 1 l, was concentrated by centrifugation to a final volume of 1.5 ml.

Soil and water analyses and enumeration of culturable bacteria

Nutrient analyses and pH measurements to the soil and water samples were done as described by Hernesmaa *et al*. (2005) and Haukka *et al*. (2005). For enumeration of culturable bacteria TGY (tryptone-glucose-yeast), R2A (APHA 1998) and chitin agar (Sarby 1992) all contain-

ing 100 μg ml^{-1} cycloheximide to prevent fungal growth were used. Soil bacteria were extracted and diluted for plating as described by Hernesmaa *et al.* (2005). Water samples were plated without dilution. The plates were grown in the dark at room temperature for 6 d (TGY and R2A) or 12 d (chitin agar).

DNA extraction and PCR amplification

DNA was extracted from 0.5 ml of concentrated water and from 0.4 g ww soil using the beadbeating technique as described previously (Haukka *et al.* 2005, Hernesmaa *et al.* 2005). From the purified DNA the 16S rRNA gene fragments of actinobacteria (approx. 1135 bp) were amplified using actinobacteria-specific forward primer F243 and general eubacterial reverse primer R1378 (Heuer *et al.* 1997). The PCR reaction contained: 2–10 ng of template DNA, 5 pmol of both primers, 2.5 μl of 10% (wt/vol) bovine serum albumin,1 μl of 10 mM dNTP solution, 5 μl of PCR buffer (10 × buffer for DynaZyme DNA Polymerase, Finnzymes), 1 μl (2 U) of DynaZyme DNA Polymerase and sterile water up to final volume of 50 μl. Amplification was performed with Gene Cycler™ (Bio-Rad) with an initial step at 94 °C for 5 min followed by 35 cycles of 1 min at 94 °C, 1 min at 62 °C, 2 min at 72 °C and the final extension at 72 °C for 10 min. The PCR products were checked in ethidium bromide stained 1% agarose gels and used for nested PCR-amplification with a GC-clamped eubacterial forward primer F968GC (Nübel *et al.* 1996) and the reverse primer R1378 to obtain a fragment of approx. 430 bp. The reaction consisted of 0.5–1 μl of the template DNA (unpurified product from the actinobacteria-specific amplification), 10 pmol of both primers, 1 μl of 10 mM dNTP solution, 5 μl of PCR buffer (10 × buffer for DynaZyme DNA Polymerase, Finnzymes), 1 μl (2 U) of DynaZyme DNA Polymerase and sterile water up to the final volume of 50 μl. An initial step at 94 °C for 5 min was followed by 35 cycles of 1 min at 94 °C, 1 min at 53 °C, 1 min at 72 °C followed by final extension at 72 °C for 10 min. The PCR products were checked as before and further analyzed in DGGE gels.

Mycobacteria-specific primers for a 16S rRNA gene fragment were designed. The forward primer Fmyco987GC (5´-GC clamp-GGGTTT-GACATGCACAGGACG-3´) was chosen by manual comparison of sequences from the EMBL databank. The reverse primer Rmyco1378 was the same as in the actinobacteria-specific PCR with one exception (5´-CGGTGTGTACAAS-GCCCGGGAACG-3´, where S is G or C). The obtained fragments were of approx. a size of 410 bp. PCR reaction contained 10–25 ng of template DNA, 10 pmol of Fmyco987GC and Rmyco1378 primers, 1 μl of 10 mM dNTP solution, 5 μl of PCR buffer (10 × buffer for DynaZyme DNA Polymerase, Finnzymes), 0.5 μl (2 U) DynaZyme DNA Polymerase and sterile water up to the final volume of 50 μl. An initial step at 94 °C for 5 min was followed by 35 cycles of 1 min at 94 °C, 1 min at 63 °C, 2 min at 72 °C and the final extension at 72 °C for 10 min.

Denaturing gradient gel electrophoresis (DGGE) and sequencing

DGGE buffers and gels were prepared following the manual for the Dcode™ Universal Mutation Detection System (Bio-Rad). Polyacrylamide concentration of the gels was 6% and the formamide-urea gradient 40%–55%. Gels poured with Bio-Rad Gradient Delivery System (Model 475) were allowed to polymerize overnight. Each well was loaded with 20 μl of PCR product and the gel was run for 4 h 45 min at 60 °C at 150 V. The same gradient and conditions were used for actinobacterial and mycobacterial fragments. After the run the gels were stained with SYBR-Green (SYBR™ Green I Nucleic Acid Gel Stain solution, FMC Bio Products; dissolved 1:10 000 in 1 × TAE-buffer) and recorded under UV light with a Fluor-S™ MultiImager system (Bio-Rad).

Major bands were excised from the DGGE gels and reamplified with primers F968 and R1378 or Fmyco987 and Rmyco1378 without GC-clamps. The PCR products were sequenced and the obtained 350–390 bp long sequences aligned using ClustalW and compared to the sequences in the EMBL as in Haukka *et al.* (2005). The obtained sequences were deposited

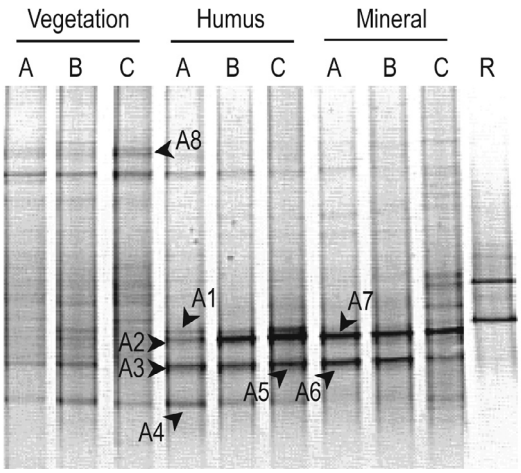

Fig. 1. DGGE gel of PCR products obtained from different layers of forest soil around lakes Ahvenlammi and Sammalisto using primers targeting actinobacterial 16S rRNA gene. Three replicates (A–C) are shown for each layer, sampled in 1997. Numbered bands (A1–A8) were sequenced. R = reference DNA from soil streptomycete isolates 52 and 63.

into the EMBL databank under accession numbers AJ488914 through AJ488925.

Results

Actinobacterial community structure

When the PCR-amplified 16S rRNA gene fragments from the humus and mineral soil layers of the forest soil were resolved in a DGGE gel, two dominant actinobacterial DNA bands (A2, A3) were obtained from each sample (Fig. 1). Two additional bands (A1, A4) were strong in the humus layer and faint in the mineral soil. DGGE patterns from the vegetation layer showed the same bands as the humus and mineral soil, but there was a multitude of additional bands above the major ones. The DGGE patterns from each soil layer were similar in 1997 and in 1998. The sequences obtained from the dominant bands of the soil DGGE patterns (A1–A8 in Fig. 1) represented members of the genus *Mycobacterium* with one exception. The only non-mycobacterial sequence (from the band A8) was 99% similar to a clone sequence of an unidentified bacterium from Finnish soil (Table 1).

DNA from water of the lakes Ahvenlammi and Sammalisto shared four to six dominant bands, obtained with the actinobacteria-specific

Table 1. Sequence comparisons of the bands excised from DGGE gels. Sequences A1–A8 were obtained using 16S rRNA gene targeted primers specific to actinobacteria (Fig. 1) and sequences M1–M6 with primers specific to mycobacteria (Fig. 3).

Sequence	Accession no.	Cluster	Closest similarity to species in the databank*	
From soil:				
A1	AJ488920	I	97%	*M. senegalense*, *M. porcinum*, *M. septicum*, *M. fortuitum*, *M. cookii*, *M. flavescens*, *M. farcinogenes*
A2	AJ488921	I	97%	*M. senegalense*, *M. porcinum*, *M. septicum*
A7	AJ488924	I	98%	*M. senegalense*, *M. porcinum*, *M. septicum*, *M. fortuitum*, *M. cookii*, *M. flavescens*, *M. farcinogenes*
M1	AJ488914	II	99%	*M.* sp. IMVS B76676 (AF016407)
M2	AJ488915	II	99%	*M. cookii* ATCC49103^T (AF480598)
A3, A5, A6	AJ488923	II	99%	*M.* sp. IMVS B76676, *M. cookii* (as above)
A4	AJ488922	II	98%	*M. cookii*, *M.* sp. IMVS B76676, *M. manitobense*, *M. terrae*
M3	AJ488916	III	100%	*M. elephantis* NRCM 01-17 (AF385898)
A8	AJ488925		99%	uncultured soil bacterium (AJ318777)
From water:				
M4	AJ488917	IV	99%	*M. fallax* ATCC35219^T (AF480600)
M5	AJ488918	IV	99%	*M. fallax* (as above), *M. chubuense*, *M. petroleophilum*, *M. phlei*, *M. fortuitum*, *M. chlorophenolicum*
M6	AJ488919	V	98%	*M. fallax*, *M. flavescens*

* Strain and accession number given for most of the 99% or 100% matches.

primers. The prevalence of these bands varied with the depth of water and season (Fig. 2). The DGGE patterns obtained with the DNA extracted from the different water layers of the two lakes were rather similar in 1997 and 1998 indicating stable actinobacterial populations. However, the hypolimnion in 1997 and the thermocline in 1998 of Lake Ahvenlammi showed one dominant actinobacterial band, which was not found in the other layers or in Lake Sammalisto. The banding patterns obtained from the two lakes were different from those obtained from the DNA of the surrounding forest soil indicating that the lake actinobacterial populations differed from those in the soil. Therefore, the actinobacterial population in water was not simply a reflection of the bacteria from the surrounding forest soil.

We found *Streptomyces* sp. by plate counting of the soils, in the order of 10^5 cfu (colony forming units) g^{-1} in vegetation and humus layers and 10^4 cfu g^{-1} in mineral soil. However, in the DGGE patterns none of the obtained major bands matched with the *Streptomyces* strains 52 and 63 isolated from the study site and used as reference DNA (Figs. 1 and 2). We tested the DNA extraction used and found it efficient in extracting different amounts of *Streptomyces* culture spiked into the soil. Therefore the absence of *Streptomyces* in the banding patterns was not a bias of the extraction protocol, but rather indicates an overrepresentation of *Streptomyces* on culture plates. The bacterial counts from soil gave 10^7 cfu g^{-1} for vegetation layer, 10^6 cfu g^{-1} for humus layer and 10^5 cfu g^{-1} for mineral soil. The counts from water gave 10^3 cfu ml^{-1} for Ahvenlammi and 10^2 cfu ml^{-1} for Sammalisto. The latter values were low probably due to a great proportion of very slow-growing or thus far uncultivated actinobacteria in the total bacterial population.

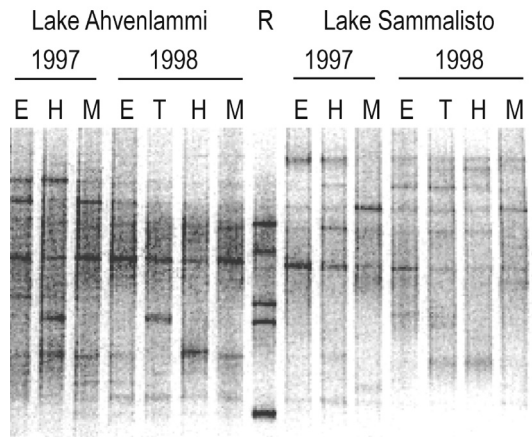

Fig. 2. DGGE gel of PCR products obtained from the lakes Ahvenlammi ja Sammalisto using primers targeting actinobacterial 16S rRNA gene. Samples were collected from the thermally stratified epilimnion (E), thermocline (T) and hypolimnion (H) and from the mixed (M) autumn water. R = reference DNA from *E. coli*, *Micrococcus roseus*, soil *Streptomyces* isolates 52 and 63, and *Frankia* AIPs1, from top to bottom.

Mycobacterial community structure

Because the dominant actinobacteria in the boreal environments belonged to the genus *Mycobacterium*, mycobacteria-specific primers were designed to study the mycobacterial communities more closely. The primer-set specificity was confirmed with various target and non-target strains (Table 2). The mycobacterial community was different in the soil and in the lakes (Fig. 3). DNA extracted from the humus and mineral soil shared three dominant bands, whereas the vegetation layer showed additional strong bands. The DGGE banding patterns obtained from the soils using the primer pair targeted to mycobacteria were similar, although not identical, to those obtained with the actinobacteria-specific primers (Figs. 1 and 3). This shows that mycobacteria were the dominating actinobacteria in the soils. No bands of the non-mycobacterial actinobacteria (e.g. A8 in Fig. 1) were obtained when mycobacteria-specific primers were used, but instead a few additional mycobacterial bands were obtained. Sequence comparison confirmed the similarity of the major bands, namely of the fragment A6 (Fig. 1) with M1 (Fig. 3) and of A4 (Fig. 1) with M2 (Fig. 3).

The DGGE patterns obtained with mycobacterial primers using DNA extracted from Lake Ahvenlammi showed six major bands shared by all layers of the water column, whereas DNA extracted from Lake Sammalisto showed only three of the bands present in Lake Ahvenlammi (Fig. 3). In water, mycobacteria were not the dominant actinobacteria as in soil, since the banding patterns obtained with the differ-

ent primer sets were different (Figs. 2 and 3). A mixture of DNA isolated from laboratory grown *M. chelonae*, *M. terrae*, *M. chlorophenolicum* and *M. phlei* representing typical environmental mycobacteria was used as a reference in a DGGE gel but no match was detected between the banding patterns of the soil or water and these species (Fig. 3).

One to 16 differing nucleotide sites were observed between the mycobacterial sequences in the sequence alignment. The similarity of the mycobacterial sequences A1–A7 and M1–M6 to 16S rRNA gene sequences available in the databank is shown in Table 1. Our sequences formed five clusters (Table 1) based on their nucleotide similarity. Clusters I and V were not closely related to the known species. Cluster II was 99% similar to *M. cookii* ATCC49103^T and *M.* sp. IMVS B76676. Cluster III was identical to a clinical isolate of *M. elephantis* NRCM 01-17. Cluster IV was 99% similar to *M. fallax* ATCC35219^T. Sequences in the clusters I, II and III were obtained from soil and those in the clusters IV and V from water. Thus sequencing of the bands confirmed that the mycobacteria dominating in the lakes differed from those prevalent in the surrounding forest soil.

Table 2. Bacterial strains used for testing specificity of the primer pair Fmyco987GC and Rmyco1378. + = amplification product obtained with the primer pair, – = no product.

Strain	
Mycobacterium abscessus MV2607	+
M. aichiense DSM 44147^T	+
M. branderi ATCC 51788	+
M. celatum ATCC 5130	+
M. chelonae abscessus NCTC 10882	+
M. chelonae chelonae ATCC 14472	+
M. chlorophenolicum CG-1M	+
M. chlorophenolicum PCP-1^T	+
M. fortuitum DSM 43528	+
M. gilvum MV2667	+
M. heidelbergense MV2494	+
M. intermedium DSM 44049^T	+
M. komossense ATCC 33013^T	+
M. murale MA 112-96^T	+
M. obuense MV2648	+
M. phlei DSM 43239^T	+
M. parafortuitum DSM 43528^T	+
M. terrae DSM 43227^T	+
Arthrobacter globiformis ATCC 8010^T	–
Comamonas acidovorans ATCC 15668^T	–
Comamonas testosteroni ATCC 11996^T	–
Escherichia coli K-12	–
Frankia sp. Bp5	–
Frankia sp. Ps1	–
Micrococcus roseus Amp733	–
Pseudomonas fluorescens HAMBI 16	–
Streptomyces clavuligerus DSM 41253	–
Streptomyces griseus DSM 40236^T	–
Streptomyces sp. 52*	–
Streptomyces sp. 63*	–

^{T} = T-type strain.
* Isolated from Nastola soil.

Discussion

We used DGGE with two nested primer sets targeting the 16S rRNA gene to amplify bacterial DNA from Finnish boreal forest soil and lake water. DGGE is an established technique for DNA-based analysis of microbial community structure and its temporal and spatial changes (Muyzer *et al.* 1998). The dominant actinobacterial and mycobacterial populations in the boreal forest soil were shown to be different from the populations in the two lakes in the catchment area. The study showed that the actinobacterial populations were slightly different and mycobacterial populations clearly different in the lakes Ahvenlammi and Sammalisto. This may reflect the different humus-content of the lakes, Sammalisto has humic and Ahvenlammi clear water. For the two years studied, the actinobacterial and mycobacterial populations remained fairly stable in each lake and in each water layer. Previously mycobacteria have been found in natural and processed waters and there is evidence that water may be the vehicle by which mycobacteria infect the human body (Wallace *et al.* 1998). It has been postulated that in Finland acid peatlands could be a source for leaching of mycobacteria to waters (Iivanainen *et al.* 1993, 1999). Iivanainen *et al.* (1993) showed that CFU count of mycobacteria in water increased during rainy periods. During our study period, summer 1998 was rainy and leaching from the forest soil into the lake water was probable. Nevertheless, no mixing of the actinobacterial and mycobacterial populations between the soil and lake water was seen in the DGGE patterns. This conclusion was confirmed

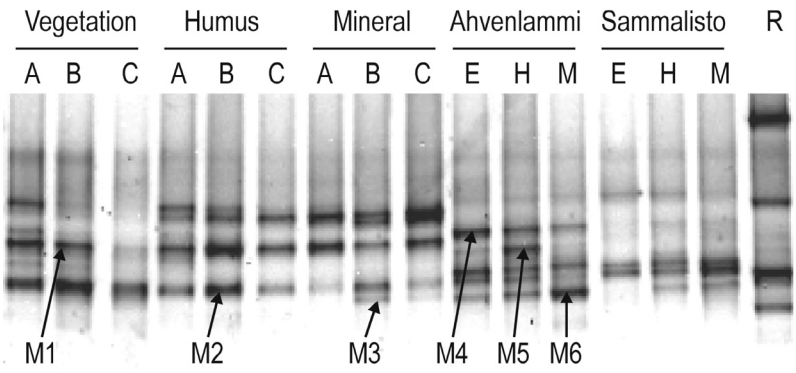

Fig. 3. DGGE gel of PCR products obtained from forest soil and lake water using primers targeting mycobacterial 16S rRNA gene. Each soil layer was analysed from three replicate samples (A, B, C). Samples from the lakes Ahvenlammi and Sammalisto were from epilimnion (E), hypolimnion (H) and mixed (M) autumn water, from year 1997. Numbered bands (M1–M6) were sequenced. R = reference DNA from mycobacterial isolates of *M. chelonae*, *M. terrae*, *M. chlorophenolicum* and *M. phlei*, from top to bottom.

by sequencing. Thus, our results do not indicate proliferation of mycobacteria in water.

The DNA-based method used in this study confirmed the reports on the common occurrence of mycobacteria in Finnish conifer forest soils based on cultivation methods (Iivanainen *et al*. 1993, 1999a, 1999b, Iivanainen 1995). Our DGGE analysis showed that mycobacteria were the dominant actinobacteria in soil. Earlier *Streptomyces* have been believed to be the most abundant group of actinobacteria in soils (Williams *et al*. 1983, Atlas and Bartha 1998). We also obtained *Streptomyces* colonies by plating, but with DGGE profiling and sequencing of the DNA from the boreal forest soil we did not detect any of them. Thus *Streptomyces* sp. did not appear to be among the dominant actinobacteria in our soils. Instead, *Streptomyces* sp. may be over-represented in plate counts as it forms aerial spores. Even one aerial hyphus may result in a large number of colonies when it is disintegrated during the sample treatment protocol. However, the PCR-based methods such as DGGE are also prone to biases, e.g. preferential amplification of certain sequences or reduction of amplification efficiency due to inhibitors such as humic material (Wintzingerode *et al*. 1997). Yet, the indication of our study that in natural fresh waters other actinobacteria than genus *Mycobacteria* dominate is supported by other studies (Warnecke *et al*. 2004, Haukka *et al*. 2005).

Several members of opportunistic pathogenic environmental mycobacteria have been found in boreal environments (von Reyn *et al*. 1993, Katila *et al*. 1995, Kirschner *et al*. 1999). Kirschner *et al*. (1999) reported the humic and fulvic acids to stimulate growth of *M. avium* and *M. intracellulare* and it is possible that also other mycobacteria are stimulated. In addition, their pH optimum for growth is between 4.5 and 5.5 and they can grow at microaerobic conditions (Primm *et al*. 2004). Our study site represented typical boreal forest environment, where average pH of the soil ranged between 4.4 and 5.1 and average pH of the lake water between 5.7 and 5.8. The physiological properties of mycobacteria seem to make them ideally suited for life in boreal conditions as well as to enhance their potential as intracellular pathogens (Primm *et al*. 2004).

Only one of the sequences we obtained from the boreal soil and lake water samples had an exact match in the databank, that was to a *M. elephantis* isolate from a human infection. Interestingly the type strains of the species which had 98%–100% sequence similarity to the sequences obtained from the coniferous forest soil were human clinical isolates: *M. septicum*, *M. fortuitum*, "*M. manitobense*", *M. terrae* and *M.* sp. IMVS B76676, or of animals: *M. flavescens*, *M. senegalense*, *M. porcinum*, *M. farcinogenes* and *M. elephantis* (according to DSMZ — Deutsche Sammlung von Microorganismen und Zellkulturen GmbH, Bacterial nomenclature up-to-date at http://www.dsmz.de/bactnom/bactname.htm). *M. cookii* was an exception being an isolate from *Sphagnum* moss. Our water sequences were

98%–99% similar to species described from environmental samples: *Mycobacterium fallax* (water), *M. chubuense* (garden soil) and *M. chlorophenolicum* (lake sediment), and only 97% similar to clinical isolates.

One of the dominant mycobacterial sequences (in cluster IV) detected in our study was 99% similar to *M. chlorophenolicum*, a species isolated and described from Finnish boreal forest environment (Häggblom *et al.* 1994). Sequences in clusters I and II were 97%–99% similar to two tentative new species isolated from Finnish stream waters (accession numbers AJ748836 and AJ748837) as well as to many other previously described species. Close sequence matches to other mycobacteria isolated and described from Finnish boreal forest environment, *M. xenopi*, *M. botniense* (Torkko *et al.* 2000), *M. bohemicum* (Torkko *et al.* 2001), and *M. palustre* (Torkko *et al.* 2002), were not found in this study. Based on the sequence similarity, the sequences in the cluster II appeared to belong to slow-growing and the sequences in the clusters III and IV to fast-growing mycobacteria (Devulder *et al.* 2005).

The mycobacteria appear to occur commonly in Finnish soil and lake water. The mycobacteria-specific PCR-primers developed in this study can help in investigating the spreading and health significance of this genus. Water has gained much attention as a source for environmental mycobacterial infections, but in the light of our results the role of soil deserves further examination. For example, peat is commonly used in animal shelters and in horticulture leading also to human exposure.

Acknowledgements: We thank Heli Karjalainen for assistance in collecting the samples, Eija Kolmonen for producing the mycobacterial sequences and Liisa Maunuksela for the *Frankia* strains used in primer testing. The sequence analysis programs were provided by the CSC-Center for Scientific Computing, Finland. This study was part of the FIBRE — Finnish Biodiversity Research project — coordinated by the Academy of Finland. The Academy of Finland grants 395902 to K. Haahtela, 395901 to K.S. and 52798 and 53305 to M.S.-S. are acknowledged.

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