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Molecular evidence for the presence of novel actinomycete lineages in a temperate forest soil

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PCR primers were designed to selectively recover partial (~ 1100 bp) actinomycete 16S ribosomal DNA sequences from a temperate forest soil. A gene library was made and colony PCR was used to identify clones containing inserts. Unique clones were identified and partial or complete insert sequences were determined for 53 clones. Phylogenetic analyses revealed that 46 (87%) of the clones sampled contained 16S rDNA sequences which fell within the actinomycete radiation. The largest group of 34 sequences formed two closely related monophyletic groups in the 16S rRNA tree, which in turn formed a weakly supported sister group with the sequence from *Actinomadura madurae*. Four novel 16S rDNA lineages were detected in *Mycobacterium*, one in *Propionibacterium* and one in *Corynebacterium*. Three novel sequences weakly grouped with *Sporichthya polymorpha*. Two sequences formed an isolated lineage not closely related to any of the reference actinomycetes. Our results lend strong support to the hypothesis that cultured (and sequenced) actinomycetes do not adequately describe the diversity of this group in the environment.

Keywords: actinomycete diversity; 16S rDNA sequences; non-culture methods; phylogenetic analysis; novel lineages

Introduction

The actinomycetes are a well-studied, phylogenetically diverse group of Gram-positive bacteria which produce a wide variety of useful products, including antibiotics, anti-tumour agents, enzymes, enzyme inhibitors and immunomodifiers [7]. New species of actinomycetes [22,46] and new compounds produced by actinomycetes are continually being identified [26,45,48]. Some actinomycetes are human pathogens (*Actinomyces, Corynebacterium, Mycobacterium*) while others infect plants (eg *Streptomyces scabies*). Actinomycetes play an important role in decomposition and degradation and *Frankia* can form nitrogen-fixing associations with woody plants. Hence there is widespread interest in the ecology, diversity and evolution of this group [10].

Traditional approaches to the identification of actinomycetes have relied on selective isolation, followed by a battery of physiological and chemotaxonomic tests to define taxonomic groupings [19]. However, it has been suggested that isolation media provide only partial answers to questions of bacterial diversity *in situ* as they will tend to recover actinomycetes which are best adapted to grow on isolation plates [54]. Under these conditions it is possible that important ecological groups may go undetected [3,50]. Molecular biology has now provided an approach for analysing natural microbial communities without culture [15,35]. The most useful gene for this purpose to date is that for 16S ribosomal RNA (rRNA) as it contains highly variable regions of sequence as well as highly conserved regions which are present in all living organisms [55]. Conserved regions are useful as target sites for the design of general probes or PCR primers whereas variable regions can be used for the design of specific probes [31] or primers. Sequence alignment and analysis are relatively straightforward and public domain databases [30] provide a valuable and cumulative resource for comparative analyses.

Results from recent molecular ecological studies of samples from different habitats have consistently revealed large numbers of novel 16S rRNA sequences [1,2,6,8,33,36,47]. The inference is that there are many previously uncultured or unsequenced bacteria still to be discovered in natural samples. Several of the published studies investigating different habitats have reported actinomycete 16S rRNA sequences. Britschgi and Fallon [2] recovered three actinomycete sequences from an anaerobic, fixed-bed, activated charcoal cyanide biotreatment column. Fuhrman *et al* [14] recovered one actinomycete sequence from a 10-m depth sample in the Atlantic ocean near Bermuda and two from a 100-m depth sample from the Pacific ocean. Schuppler *et al* [40] recovered several nocardioform actinomycete and mycobacterial sequences from sewage. Several clones from a soil sample from a paddy field were recently found to be related to the actinomycetes at a deep level and two clones were recovered within the actinomycete radiation, one clustering within the *Streptoverticillium/Streptomyces* group and one clustering next to *Corynebacterium mediolanum* (Ueda, unpublished data available through the RDP [30]). The latter four studies used general bacterial primers to determine bacterial diversity, ie they were not trying to selectively recover actinomycete 16S rRNA sequences. In contrast, Stackebrandt and colleagues investigating an Australian soil sample, used one PCR primer designed to specifically recover streptomycete-like sequences in combination with a more general bacterial primer [42]. Their aim was to selectively enrich

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for streptomycete sequences; an actinomycete group which is considered to be particularly important in soil. Interestingly, only two streptomycete sequences were recovered and these were different from the sequences of the 50 streptomycete isolates which could be cultured from the same soil sample [42].

In the present investigation we sampled the phylogenetic diversity of actinomycete 16S rDNA in a soil sample from a mature and undisturbed deciduous woodland, using PCR primers designed to enrich for sequences from these organisms. The results of this study provide further evidence that an extensive resource of apparently untapped actinomycete diversity exists in nature.

Materials and methods

Sample collection

Soil material at a subsurface depth of 5–20 cm was collected from a single site beneath a beech tree in June 1992 and stored at −70°C until used for DNA extraction. The soil sample had a pH of 5.2 and contained 11.9% organic carbon and 0.70% nitrogen, as determined by combustion analysis using a Carlo Erba 1106 analyser. Inorganic particle analysis was performed using a modification of the pipette method [5]. The sample contained 0.67% gravel; 2.29% coarse sand (600 μm); 2.83% medium sand (212 μm); 22.37% fine sand (63 μm); 34.54% coarse silt (0.02 μm); 13.58% medium silt (0.006 μm), 8.56% fine silt (0.003 μm) and 15.05% clay. The humic content of the soil was 7.5% calculated using the method of Mehlich [32].

DNA extraction

Total DNA was extracted from 0.5 g of the soil sample using a bead beater method since this lyses actinomycete cells efficiently in our hands. Prior to extraction the soil was washed with 120 mM sodium phosphate buffer (pH 8.0). The following ingredients were added to a sterile 2-ml screw cap Eppendorf tube: 500 μl of glass beads (0.17–0.18 mm diameter); 500 μl phenol/chloroform/isoamyl alcohol (IAA) (24 : 24 : 1); 750 μl of 120 mM phosphate buffer pH 8.0 and 0.5 g of washed soil. The tube contents were bead beaten at 2000 rpm for 1 min, the glass beads were separated by centrifugation at 10 000 × *g* for 10 min and the top phase was removed to a sterile Falcon tube and placed on ice. Additional phosphate buffer (750 μl) was added to the soil mix and the bead beating was repeated for 30s. The centrifugation step was repeated and the two top phases were combined. This was phenol-extracted once using an equal volume of Tris-equilibrated phenol, then once with phenol/chloroform/IAA, followed by one chloroform/IAA extraction. One-tenth volume 5 M NaCl plus one volume 30% PEG 8000 [28] were added to the final top layer and the tube placed on ice for 15 min. The sample was centrifuged at 10 000 × *g* for 20 min and the supernatant phase was discarded and the pellet was resuspended in 50–100 μl TE pH 8.0. The DNA sample was then electrophoresed on a 3% NuSieve agarose gel and the high molecular weight DNA band was excised. Approximately 2 μg of DNA was obtained per g of soil.

Primer design

Actinomycete 16S rRNA sequences are quite diverse and stretches of sequence unique to the entire group are rare, especially if one includes some of the deeper branches related to taxa such as *Atopobium* and strain TH3 [10,42]. Each PCR primer was therefore designed based on the presence of a string of sequence present in >90% of the approximately 250 actinomycete sequences available for study [30,31], and concentrating on the main actinomycete radiation ie those taxa traditionally considered as actinomycetes [10]. The PCR primers are as follows: ACT283F (5′-GGGTAGCCGGCCUGAGAGGG) corresponding to *E. coli* 16S rRNA positions 283 to 302 [4] and ACT1360R (5′-CTGATCTGCGATTACTAGCGACTCC) corresponding to the complement of *E. coli* 16S rRNA positions 1360 to 1336 [4]. Both primers were designed as single sequences although sequences from some actinomycete species differed from one primer by a single base (Table 1). PCR conditions were optimised so that product was still obtained from *Actinomadura madurae* NCTC 5654, which had a single base mismatch for the forward primer. A small sample of non-actinomycete species was used as negative controls (Table 1) to check for non-specific amplification during the optimisation experiments.

Amplification of actinomycete 16S rDNA from forest soil DNA

The excised agarose slice containing high molecular weight DNA from the soil sample was melted at 65°C for 15 min, vortexed, and serial dilutions in the range 1/10 to 1/100 prepared. One microlitre from each dilution was used for replicate PCR reactions containing 20 pmol of each primer; 125 μmol each dNTP; 1 unit of Taq polymerase in 1× buffer (670 mmol Tris pH 8.8 and 20 mmol $MgCl_2$) in volumes of 50 μl. A hot start was used [21] and thermal cycling conditions were: 10 cycles denaturation at 94°C for 1 min, primer annealing at 65°C for 30 s, extension at 72°C for 2 min, followed by 20 cycles denaturation at 92°C for 30 s, primer annealing at 65°C for 30 s, extension at 72°C for 2.5 min, followed by a final extenson step at 72°C for 5 min. Aliquots (30 μl) from 15 separate PCR reactions were combined in an attempt to reduce the effects of potential amplification biases such as PCR drift [51] which may have occurred in individual reactions.

Actinomycete 16S rRNA library construction

The pooled PCR products were gel purified by electrophoresis in 1% agarose, the DNA band was extracted using Qiaex resin (Qiagen Ltd, Surrey, UK) and ligated into the pGEM-T vector according to the manufacturer's instructions (Promega Ltd, Southampton, UK). Ligation products from three reactions were pooled and used to transform supercompetent Epicurian Coli XL1-Blue MRF′ cells according to the manufacturer's instructions (Stratagene Ltd, Cambridge, UK).

Screening of the 16S rDNA library

Individual colonies were screened for the presence of inserts of the expected size using a colony PCR method developed by Mark Munson (NHM and University of Essex, personal communication) in microtitre well plates in

Table 1 Actinomycete-specific forward and reverse primer sites on 16S rDNA at positions 283 to 302 and 1336 to 1360 (*E. coli* numbering) respectively

Forward primer target sequence		Reverse primer target sequence	
283 302	**Actinomycetes**	1336 1360	
5′- G G G T A G C C G G C C T G A G A G G G	250 Species	5′- G G A G T C G C T A G T A A T C G C A G A T C A G	
. . . . . A	*Amad. madurae*	. . . . .	
. . . G	*Frankia* sp	. . . . .	
	Reference bacteria		
C C C . . . T . . T . . . . . A	*E. coli*	. . . A . . . . . T G	
C . . . . . T . . T . . . . . A	*Azs. brasiliense*	. . . A . . . . . G	
. . T . . . T . . T . . . . . A	*Dsb. latus*	. . . A . . . . . G	
. . . . . T . . . . . A	*Dsv. vulgare*	. . . A . . . . . T C G	
C A . . . . T T . . T . . . . . A	*Nmn. europaea*	. . . A . . . . . G	
. . . . .	*Fus. alocis*	. . . A . . . . . G A	

Base positions which are the same as the primer target sequence are represented by dots. *Amad. madurae – Actinomadura madurae*; *Azs. brasiliense – Azospirillum brasiliense*; *Dsb. latus – Desulfobacter latus*; *Dsv. vulgare – Desulfovibrio vulgare*; *Nmn. europaea – Nitrosomonas europaea*, *Fus. alocis – Fusobacterium alocis*.

25-μl volumes using SP6 and T7 as amplification primers. PCR products from all clones with inserts were single lane sequenced [39] using cycle sequencing [9] through a variable region of the 16S molecule, corresponding to *E. coli* positions 302 to 530 [4]. G tracks were scored for the occurrence of unique clones which were then either fully or partially sequenced directly. Coverage of the library [18] was calculated using the equation $C = 1 - (n_1/N)$ [17]. This gave one indication of how thoroughly the recombination library was sampled by our clone selection and sequencing.

Phylogenetic analyses

The actinomycete 16S rDNA sequences recovered from our sample were submitted to EMBL and assigned the accession numbers Z73363 to Z73415.

Clone sequences were first analysed using the RDP SIMILARITY_RANK program [30] which identified the most closely related reference sequences in the RDP. These sequences were included in a subalignment from the RDP [30] which contained sequences from each of the main bacterial groups [55]. Cloned sequences shown to be members of the actinomycete phylum were then included in an actinomycete alignment containing approximately 400 sequences [10,30,31] to determine their detailed relationships. In each case only those segments of sequence which could be aligned unambiguously were used for phylogenetic inference. Corrected pairwise distances were generated from percent similarities using the method of Jukes and Cantor [24] and phylogenetic trees constructed by the neighbor joining method [38]. Bootstrapping (100 replicates) was used to assess support from the data for particular hypotheses of relationship [12].

All clone sequences were investigated for the presence of chimeric artifacts using the CHECK_CHIMERA program [30]. In addition, cloned sequences were separated into three sections including a 5′ end, a middle section and a 3′ end section and analysed phylogenetically. Some types of chimeras may be detected if the phylogenetic placement of a cloned sequence changes radically when different regions of the sequence are analysed [27]. Some sequences were also analysed for potential secondary structure as some chimeras may be detected by this method [17].

Results

Under the PCR conditions described, the actinomycete primers consistently amplified single PCR products approximately 1100 base pairs in length, from the forest soil DNA. Using the same stringent PCR conditions, DNA from the non-actinomycetes tested did not amplify with the specific primers, but the same DNA was amplified using general eubacterial 16S rDNA PCR primers.

Single lane G track sequences were analysed for 126 clones to identify sequence types. Fifty-three clones were directly sequenced to obtain at least 400 bases of sequence for each clone from regions equivalent to *E. coli* positions 302 to 530 or 530 to 943. The full insert sequence was determined for 24 of these clones. All sequences were checked for chimeras and within the limitations of the three months used we did not detect evidence of chimeric sequences among the clones sampled. The phylogenies generated from each of the three sets of partial sequences were similar, and base changes in stem regions usually comprised compensatory base changes. One exception was clone EpT_1.150 where a non-compensatory base change was identified at *E. coli* position 973 resulting in a potential U-U pair. This U-U pair was previously reported [14] in a marine environmental clone sequence, BDA1-5, which clustered with the actinomycete *Rhodococcus equi* in some analyses. All other published actinomycete sequences have an A at position 973 which pairs with U at position 960 on helix 35. The base pairing at this position is 80–90% conserved among eubacteria [20]. Thirteen of the 53 clones sequenced were considered to be unique under the criterion than they shared less than 97% sequence similarity to the other sequences, giving an estimated coverage of 75% [17].

Based upon an analysis of 387 sequence positions (Figure 1) the 53 soil clones examined clustered in seven groups, six of which clustered within the actinomycete phylum. One group, containing seven clones, clustered with 100% bootstrap support with the acidiphilic bacterium *Acidobacterium capsulatum* [25]. An environmental clone sequence (MC 13) from Mount Coot-tha soil in Australia [42] was also recovered within this cluster but with only 46% bootstrap support (Figure 1).

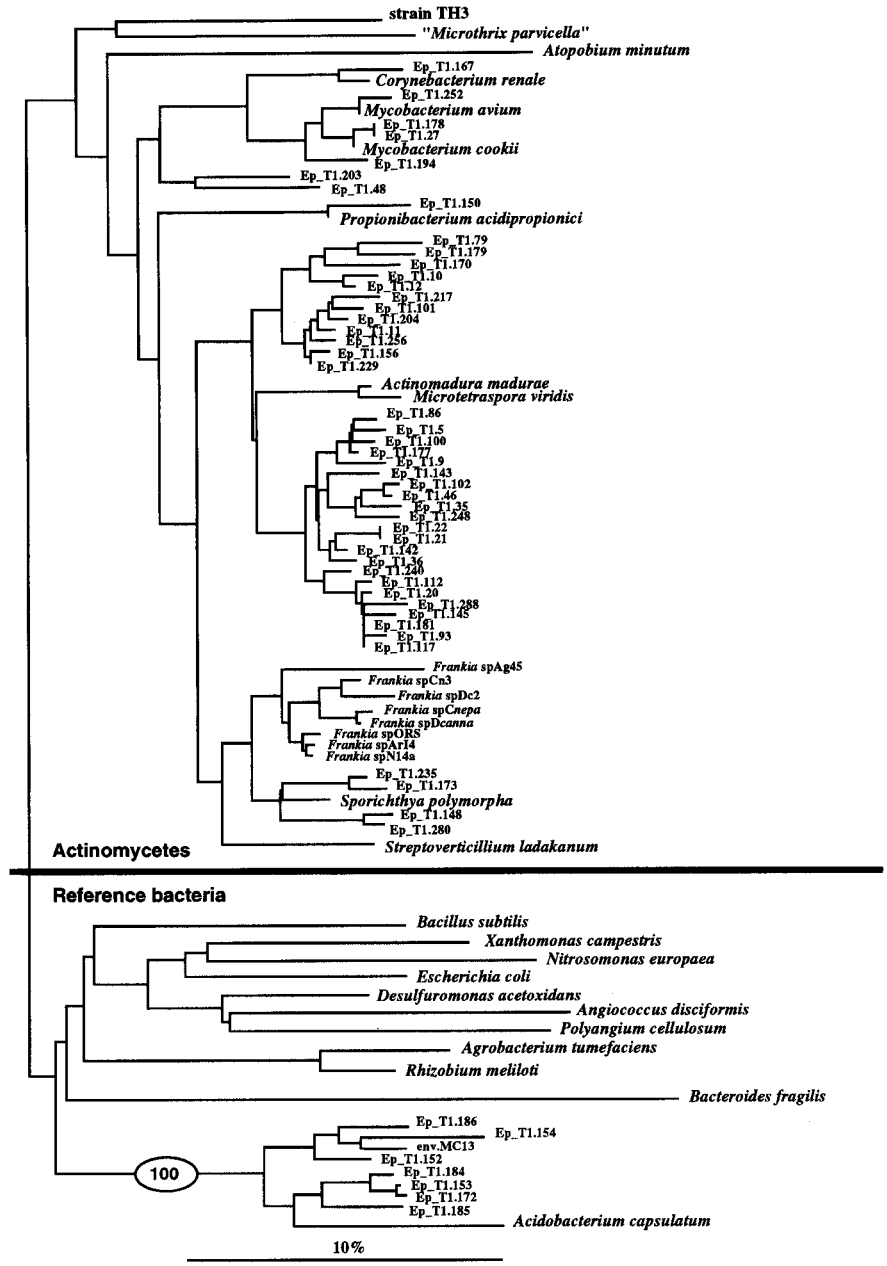

Figure 1 Phylogenetic tree generated from a selection of reference bacterial 16S rDNA sequences showing that 46 out of 53 soil DNA clones (87%) cluster within the actinomycete phylum. The analysis was based on 387 bases corresponding to an unambiguously aligned sequence from the region equivalent to *E. coli* positions 489 to 886. The scale bar represents an estimate of 10% sequence divergence. Clone sequences have the prefix EpT.

The phylogenetic relationships of selected actinomycete-like clones and reference actinomycetes, were analysed in more detail using longer stretches of sequence (Figure 2). Two monophyletic groups, each with 100% bootstrap support, contained most of the clone sequences and together formed a well-supported monophyletic group (79% bootstrap support), hereafter referred to as cluster I. In analyses of original sequence, data cluster I consistently formed a sister-group relationship with *Actinomadura madurae* and relatives, but this relationship received little support from bootstrap analyses.

A small group of soil clones (cluster II, Figure 2) formed a weakly supported cluster with *Sporichthya polymorpha* and *Frankia*. Two sequences EpT_1.48 and EpT_1.203, formed a strongly supported monophyletic group (cluster III) whose position was unstable within the actinomycete radiation.

The remaining clone sequences occupied strongly supported positions among sequences from currently recognised genera of actinomycetes. Clone EpT_1.150 was closely related to *Propionibacterium acnes* and clone EpT_1.167 to *Corynebacterium renale*. Four of the clones formed novel lineages within a well-supported *Mycobacterium* clade, for which there are published sequences for approximately 100 strains and species. The detailed relationships between slow and fast growing *Mycobacter-*

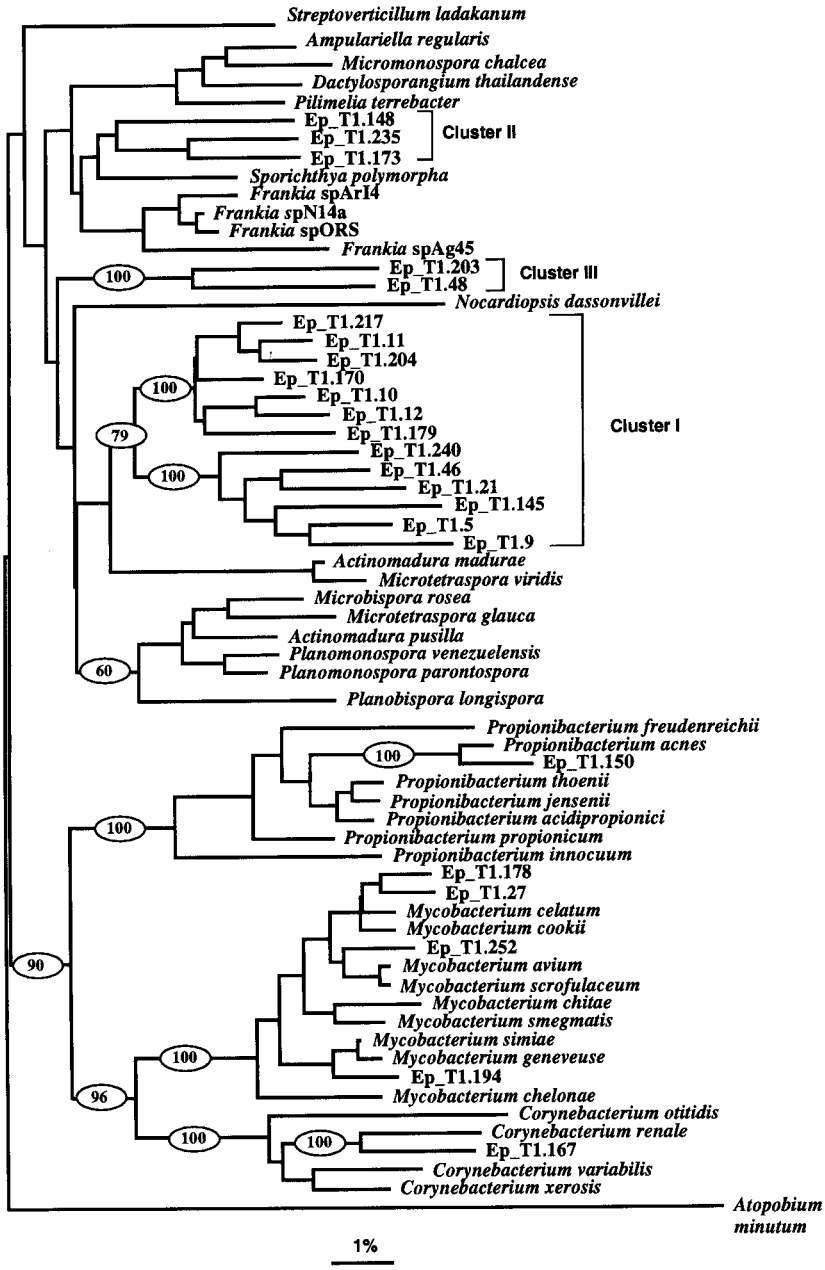

Figure 2 Phylogenetic relationships among reference actinomycetes and selected clone sequences from a temperate forest soil DNA 16S gene library. The analysis was based on 961 bases corresponding to an unambiguously aligned sequence from the region equivalent to *E. coli* positions 303 to 1335. The scale bar represents an estimate of 1% sequence divergence. The relative stability of clusters of sequences relevant to discussion are represented by bootstrap values in circles at basal nodes and are based on 100 replicates.

ium species and the clones recovered from the gene library are displayed in Figure 3. Some slow growing *Mycobacterium* species have a long helix 18 [43] and this apparent synapomorphy (shared, derived character) is also found in the three clones recovered in this portion of the *Mycobacterium* tree (Ep_T1.27; Ep_T1.178; Ep_T1.252). Clone Ep_T1.194 is closely related (97% sequence similarity) to *Mycobacterium simiae* and both have a short helix 18 [43].

Discussion

The aim of the present investigation was to develop a PCR-based strategy to recover actinomycete 16S rDNA selectively from mixed community DNA extracted from a woodland soil sample. We focused on actinomycetes because of their importance as producers of bioactive compounds [7] and the consequent interest in their natural diversity as a potential resource for exploitation, as well as their ecological importance [53,54].

In designing the experiment we tried to avoid some of the more obvious sources of potential bias. Cell lysis was performed mechanically using bead beating since this method lysed all of the different actinomycete wall types we have so far analysed. PCR amplifications were replicated and pooled prior to cloning to avoid random effects which might have occurred in any one reaction [51]. The

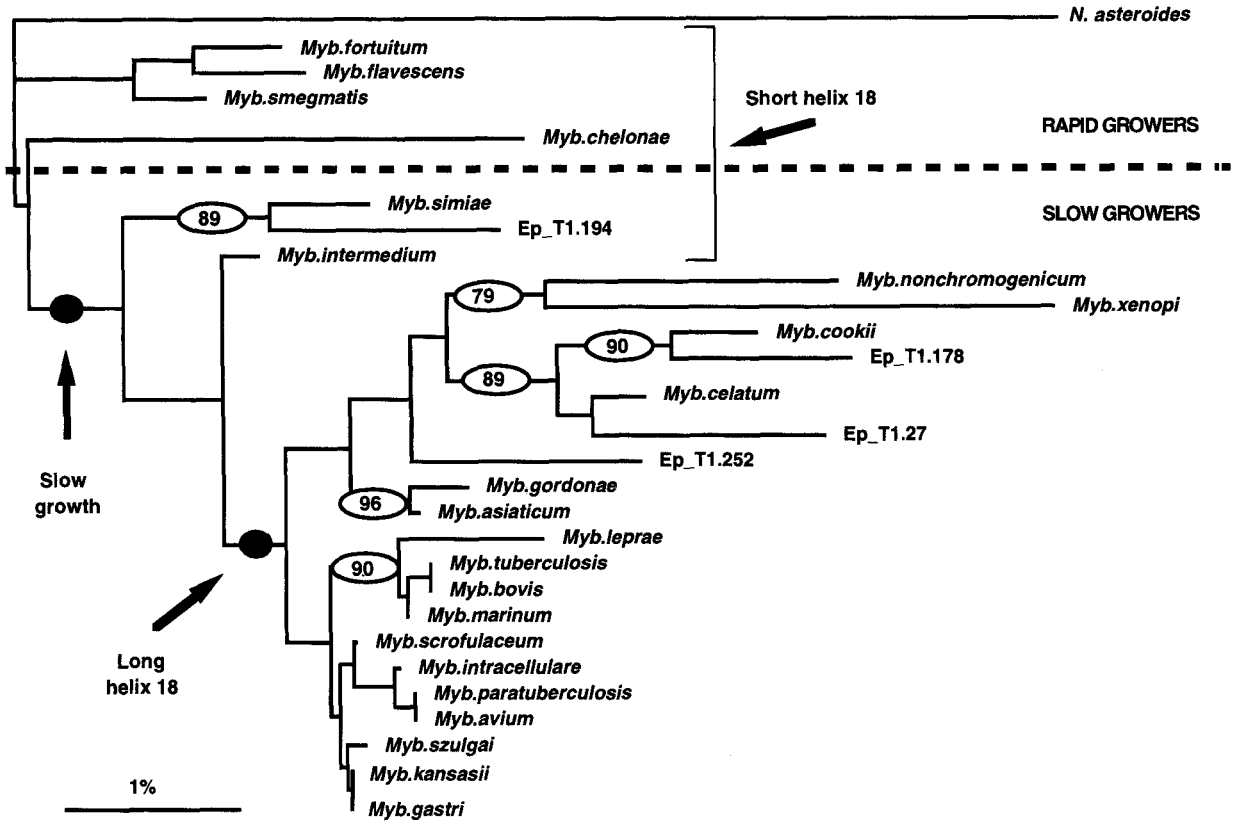

Figure 3 Phylogenetic tree showing the relationships between selected *Mycobacterium* species and four temperate forest soil clones which cluster within this genus. This tree is modified and extended from an original in Rogall *et al* [37]. The analysis was based on 1008 bases corresponding to an unambiguously aligned sequence in the region equivalent to *E. coli* positions 303 to 1335. The scale bar represents an estimated 1% sequence divergence. The relative stability of clusters of sequences are represented by bootstrap values in circles at basal nodes and are based on 100 replicates.

PCR primers were designed as single sequences since degenerate primers may produce skewed clone abundances due to primer exhaustion [17]. However, some actinomycete 16S rDNA sequences contained single mismatches relative to the primer target sequences, hence the PCR conditions were relaxed to enable amplification from these strains. Since the majority of sequences analysed from the gene library were recovered within the actinomycete phylogenetic radiation, it appears that the overall strategy was a successful one.

Before we discuss the actinomycete-like sequences it is worth considering the small cluster of non-actinomycete sequences which were also recovered. The phylogenetic position among the bacteria of this highly divergent group of sequences was not resolved in our analyses. However, they formed a strongly supported monophyletic group which also included the acidiphilic *Acidobacterium capsulatum* isolated from acidic drainage from a Japanese mine [25], and a DNA clone sequence from an Australian acid forest soil from Mount Coot-tha [42]. It has been suggested previously that the large population size of bacteria and their ease of dispersal may mean that most prokaryotes will be widely distributed [13] and that consequently endemism might be limited. Certainly in the oceans there appear to be a small number of monophyletic groups of Archaeal and Bacterial sequences, representatives of which occur in most samples to date [6,34]. The new cluster containing sequences from Australia and England provides another example of a geographically widespread monophyletic group, but this time in soil. It will be interesting to see if additional sequences from this group are found in future studies and equally important, to investigate their roles in the environment.

The majority of actinomycete clone sequences analysed were recovered in two closely related monophyletic groups, called cluster I in Figure 2. The inferred predominance of this group in our gene library may reflect the importance of this novel group *in situ*. However, further work is needed using more quantitative methods such as probing [15] to test this hypothesis. It has been demonstrated that the relative proportions of sequences recovered using PCR from mixtures of homologous genes may be biased towards particular templates [44] and that different prokaryotes may contain different numbers of rRNA genes [11]. Interestingly, we did not sample any sequences from our library which were related to *Streptomyces* although our extraction procedure releases DNA from these bacteria and the PCR primers should amplify their rDNA. This genus is often recovered on isolation plates inoculated with soil samples [53], although we have not carried out such experiments on our particular soil. A previous investigation using a *Streptomyces*-specific PCR primer recovered only two *Streptomyces* sequences among clones sampled from a soil library [42]. It remains a possibility that *Streptomyces*

sequences are present in the library we made from our soil sample, but they were not detected by our sampling. An estimate of library coverage suggests that in a similar clone library of infinite size our sampling would account for 75% of the clones. However, this gives no indication of the identities or diversity of the remaining sequences.

The relationship of novel actinomycete cluster I to other actinomycete sequences was not precisely resolved, but in most analyses of non-bootstrapped data it shared a relationship with *Actinomadura* and its close relatives. There was considerable variation within cluster I in terms of sequence divergence between individual clone sequences. At present we hypothesise that this sequence diversity is real, rather than an experimental artifact, since analysis of individual sequences using the available programmes failed to indicate chimeras. Converting the observed 16S rDNA sequence variation within cluster I (or II or III) into the familiar terms of potential novel actinomycete species or genera is not straightforward [41] but it is an interesting and illuminating excercise. The most distantly related clones are Ep_T1.145 and Ep_T1.179 which share 90.3% similarity over the fragment of 16S rDNA available for comparison. This is similar to the sequence similarity between *Propionibacterium* and *Mycobacterium* for the same segment of sequence. The most similar pairs of clones in cluster I (Ep_T1.217 & Ep_T1.11 and Ep_T1.217 & Ep_T1.204) are 98% similar to each other for this stretch of 16S sequence. Using criteria established on the basis of correlations between DNA–DNA pairing studies and those based on full length 16S rRNA sequence similarities for cultured organisms [41], each clone in cluster I potentially represents a new species to be isolated, and cluster I (and cluster II and III on these criteria) may represent one or more new genera.

Mycobacterium is one of the best studied actinomycete genera in terms of 16S rRNA sequence analysis so the detection of four novel sequences is an interesting finding. It reveals that even well-studied bacterial groups may be incompletely described by cultured or sequenced taxa. *Mycobacterium* species are commonly described from soil samples [23,49], so it is perhaps not too surprising that we detected members of this genus in our sample.

Cluster II, which comprised three novel sequences, grouped with *Sporichthya polymorpha* which has an unique life cycle among actinomycetes [29]. Although bootstrap support for this sister group relationship was low, it was recovered consistently regardless of the region of sequence analysed or the number of sequences included in the analysis. The specific relationship of the two cluster III sequences to known actinomycetes was not resolved in this analysis.

Evidence of the occurrence of novel actinomycetes in a forest soil sample is in accord with other molecular studies [1,16,52] which indicate that known cultured microorganisms may represent a small fraction of microorganisms present in nature. Actinomycetes are well known as producers of bioactive compounds and it is not unreasonable to suggest that new lineages detected by molecular studies may represent taxa which will be equally productive, since they share a common history. In order to test this hypothesis it is essential to first isolate the new actinomycetes from which the sequences originate. The existing sequence information will play an important part in this endeavour. Specific probes or PCR primers to the novel target group(s) can be used to prescreen habitats, to monitor enrichment cultures or to assess the efficacy of isolation media [54]. Exploitation of the newly discovered actinomycete diversity revealed by molecular analyses will require the skills of microbial physiologists and ecologists.

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