Current Trends in Microbiology
Vol. 5, 2009

Original Communication

The use of *trpB* gene in resolving phylogenetic diversity within the group of *Streptomyces*

Christos Meintanis[1], Kalliopi I. Chalkou[1], Konstantinos Ar. Kormas[2], Despoina S. Lymperopoulou[1], Efstathios A. Katsifas[1] and Amalia D. Karagouni[1,*]

[1]Microbiology Group, Department of Botany, Faculty of Biology, University of Athens, 15781 Athens, [2]Department of Ichthyology & Aquatic Environment, University of Thessaly, 38446 Nea Ionia, Greece

ABSTRACT

In the present work, we investigated the ability of *trpB* gene, which encodes a primary metabolism enzyme involved in the tryptophan synthesis, to be used as an alternative to 16S rRNA for sequence similarity analysis in the *Actinobacteria* group. *trpB* DNAs (504 bp) were amplified from 13 *Actinobacteria* type strains, in addition to 24 environmental streptomycete isolates with different BOX-PCR profiles. The sequences and the phylogenetic tree of *trpB* were compared to those obtained from 16S rRNA gene analysis, for the total of the examined bacteria. The results demonstrated between 93 – 100 % (16S rRNA) and 86 – 100 % (*trpB*) similarity among the examined bacteria of the genus *Streptomyces* and suggested that *trpB* sequence similarity analysis allows a more accurate discrimination of the species within *Streptomyces* genus than the more commonly used 16S rRNA. Furthermore, DGGE analysis was also applied in habitats which exhibit a high degree of streptomycete diversity. The biodiversity patterns produced led to similar estimation of diversity, whether using 16S *Actinobacteria* group specific primers or the *trpB* novel primers. In conclusion, our study suggested that *trpB* sequence similarity analysis is a powerful tool for discrimination between species within the ecologically and industrially important strains of *Streptomyces* genus.

*Corresponding author
akar@biol.uoa.gr

KEYWORDS: *Streptomycetes*, *trpB*, 16S rRNA phylogeny, diversity

INTRODUCTION

Actinobacteria, and especially the genus *Streptomyces*, are involved in important biotechnological processes such as decomposition of organic matter and xenobiotic compounds, biological control of plant pathogens and secondary metabolites production [1, 2]. Yet, taxonomic species definitions remain unresolved within the *Actinobacteria* group and questions have arisen about intraspecies diversity that could differentiate isolates with potential biotechnological importance [3].

The comparison of partial 16S rRNA sequences, including the variable γ region, has been shown to be a useful tool for distinguishing genera of the class *Actinobacteria* [4, 5, 3, 6]. However, new molecular tools are needed to establish diversity within *Actinobacteria* communities since 16S rRNA gene sequence analysis was not proposed for resolution of species within genera of this group [7, 8, 9, 10]. Heuer *et al.* [11] suggested that 16S rRNA could provide useful indications for detection of *Actinobacteria* in environmental samples. Additionally, rRNA sequences used alone can be misleading due to the presence of multiple 16S rRNA operons and the occurrence of recombination between strains of the same bacterial group [12, 13, 14].

Due to the need for development of additional approaches for taxonomic analysis of groups within the *Actinobacteria*, the sequence analysis of variable regions within "housekeeping" genes has been used [15, 14, 16]. In this study, we used *trpB*, a housekeeping gene encoding a primary metabolism enzyme involved in the tryptophan synthesis, as an alternative molecular marker to 16S rRNA gene for successfully resolving phylogenies between closely related species and subspecies within the group of *Actinobacteria*. New group specific primers for the *Actinobacteria* were developed and tested using 13 type-species representing diverse genera within the *Actinobacteria* and 24 *Streptomyces* strains isolated from greek habitats. Streptomycete isolates were selected on the basis of different BOX-PCR patterns. 16S rRNA and *trpB* genes were amplified from all studied strains and sequence similarity analysis was performed, along with the construction of phylogenetic trees for both genes. In addition to these studies, DGGE analysis in habitats exhibiting high streptomycete diversity for both genes was performed and biodiversity patterns were compared. Finally, the amplified region of the *trpB* gene studied above (504 bp) is the longest described for *Actinobacteria* in literature for application in DGGE analysis.

MATERIALS AND METHODS

Bacterial strains

The bacteria used in this study included 13 type strains representing 11 genera within the *Actinobacteria* (Table 1), obtained from DSMZ. They included *Actinoplanes phillipinensis* DSM43019, *Actinosynema mirum* DSM43827, *Cellulomonas iranensis* DSM14785, *Dactylosporangium aurantiacum* DSM43157, *Geodermatophilus obscures* DSM43160, *Micrococcus luteus* DSM20030, *Nocardia veteran* DSM40777, *Planobispora rosea* DSM43051, *Rhodococcus rhodochrous* DSM43202, *Saccharothrix syringae* DSM43886, *Streptomyces aureofaciens* DSM40127, *Streptomyces coelicolor* DSM40233, *Streptomyces lydicus* DSM40461.

The collection was supplemented with 150 *Streptomyces* strains isolated from twelve different Greek habitats [17] from our laboratory culture collection (Microbiology Lab. of University of Athens, Greece). All the above streptomycete belonged to *S. albidoflavus*, *S. cyaneus*, *S. exfoliatus* and *S. rochei*, as phenotypically characterised according to Williams *et al.* [18]. These 150 streptomycete isolates corresponded to 24 different BOX-PCR genomic fingerprints.

Table 1. Type *Actinobacteria* strains investigated in this study.

Type strain	Species	Group[a]	GeneBank accession number	
			16S rDNA	*trpB* gene[b]
DSM43019	*Actinoplanes philippinensis*	*Micromonosporaceae*	X93187	DQ192159
DSM43827	*Actinosynnema mirum*	*Actinosynnemataceae*	X84447	DQ192160
DSM14785	*Cellulomonas iranensis*	*Cellulomonadaceae*	AF064702	DQ192161
DSM43157	*Dactylosporangium aurantiacum*	*Micromonosporaceae*	X72779	DQ192162
DSM43160	*Geodermatophilus obscurus*	*Geodermatophilaceae*	X92355	DQ192163
DSM20030	*Micrococcus luteus*	*Micrococcaceae*	AJ536198	DQ192164
DSM40777	*Nocardia veterana*	*Nocardiaceae*	AF430059	DQ192165
DSM43051	*Planobispora rosea*	*Streptosporangiaceae*	AB028654	DQ192166
DSM43202	*Rhodococcus rhodochrous*	*Nocardiaceae*	X80625	DQ192167
DSM43886	*Saccharothrix syringae*	*Actinosynnemataceae*	AF114812	DQ192168
DSM40127	*Streptomyces aureofaciens*	*Streptomycetaceae*	Y15504	DQ192171
DSM40233	*Streptomyces coelicolor*	*Streptomycetaceae*	Z76678	DQ192169
DSM40461	*Streptomyces lydicus*	*Streptomycetaceae*	AB184281	DQ192170

[a]*Actinobacteria* groups are defined according to families proposed by Stackebrandt, *et al.* (1997).
[b]Numbers were obtained in this work.

BOX-PCR analysis

Bacteria were harvested from 1 ml cultures in TSB (Sharlau SA, Spain) after 48 h of incubation at 30 ^{o}C and 200 rpm. DNA extraction was performed following the protocol of Hopwood *et al.* [19]. DNA concentration was determined with a spectrofluorimeter (Hitachi U1100) and adjusted to 40 ng/µl. The BOX element (BOX1A) was amplified using the BOXA1R primer 5'-CTACGGCAAGGCGACGCTGACG-3' [20]. PCR and electrophoresis conditions were adjusted according to Rademaker and de Bruijn [21]. PCR reaction for each isolate was repeated three times for reproducibility.

16S rDNA PCR

For the amplification of the region of 16S rDNAs of the 24 isolates, primers F27 and R1492 corresponding to nucleotides 8 – 1510 of the *Escherichia coli* 16S rDNA were used. Primers and PCR conditions were described previously [22]. PCR products were purified using Nucleospin Extract® PCR kit (Macherey - Nagel, Germany) and DNA strands were sequenced commercially (www.macrogen.com).

Amplification of *trpB* gene fragments

Oligonucleotide primers to amplify a region of *trpB* gene of *Actinobacteria* were designed on the basis of published sequences by using the Hitachi Software DNASIS. PCR amplification of a 504 bp fragment of the *trpB* gene, corresponding to nucleotides 252 to 755 of *S. coelicolor* A3(2) [AF054585] *trpB* gene, was performed using the forward primer *trpB*F 5´-CCGATCTTCCTCAAGCGCG–3´ and the reverse *trpB*R 5´-GCCGATGGCGTTGGAGCC– 3´.

Amplification of the *trpB* fragment gene was performed in a PCR mix of 50 µl final volume, contained 40 ng of template DNA, 50 mM KCl, 10 mM Tris – HCl (pH 9), 0.1 % TritonX-100, 1 Unit of Promega Taq DNA Polymerase, 0.2 mM deoxynoucleotide triphosphates, 3.75 mM $MgCl_2$, 20 nM of each of the primers *trpB*F and *trpB*R and 5 % (v/v) acetamide. A 5 min initial denaturation step at 94 ^{o}C was followed by 35 cycles of amplification, consisting of 1 min denaturation at 95 ^{o}C, 1 min of primer annealing at 53 ^{o}C and 2 min of primer extension at 72 ^{o}C and finally by a 10 min final extension step at 72^{o}C. PCR products were purified using Nucleospin Extract® PCR kit (Macherey – Nagel, Germany) and both DNA strands were sequenced commercially (Macrogen, Korea).

Phylogenetic analysis

Sequence data were compiled and aligned using the ARB software (www.arb-home.de) and compared with sequences obtained from the ARB and GenBank databases. Phylogenetic analyses were performed using the neighbour-joining method as determined by distance Jukes-Cantor analysis, implemented in PAUP* [23]. Heuristic searches under minimum evolution criteria used 1000 random-addition replicates, followed by tree bisection-reconnection topological rearrangements. Bootstrapping under parsimony criteria was done with 1000 replicates for 16S rRNA data sets. Phylogenetic trees of deduced amino acid sequences of PCR-amplified *trpB* genes (ca. 500 bp) of the *Actinobacteria* strains used in this work were based on the neighbour-joining method, as determined by distance Jukes-Cantor analysis. One thousand bootstrap analyses (distance) were conducted, and percentages greater than 50 % are indicated at the nodes (Fig. 2B). *trpB* sequences retrieved from this study have GenBank accession numbers from DQ192135 to DQ192158.

DGGE analysis

Soil samples were used for total community DNA extraction using UltraClean™ Soil DNA kit (MO BIO, USA). DGGE analysis of total *Actinobacteria* community DNA from the sampling sites was performed using universal eubacteria primers F243 and R513GC (Heuer *et al.* (1997)). The GC-rich sequence is attached at 3´ end of reverse primer (R513) to prevent complete melting during separation in the denaturing gradient [11]. PCR conditions were followed as described by Heuer *et al.* [11]. PCR samples were loaded onto 6 % w/v polyacrylamide gels in 1 % TAE. The polyacrylamide gels were made with denaturing gradients ranging from 40 to 60 % (where 100 % denaturants correspond to 7 M urea and 40 % formamide). The electrophoresis was run at 60 ^{o}C for 4 h at 150 V. A routine silver staining protocol described by Riesner *et al.* [24] was used for the detection of DNA in DGGE gels.

DGGE analysis based on the selected region of *trpB* gene was performed using the same protocol with denaturating gradients rearranged to range from 40 to 70 %, the electrophoresis was run at 60 ^{o}C for 6 h at 150 V. The GC-rich sequence was attached at 5´ end of forward primer *trpB*F and PCR conditions were as described above for primers *trpB*F and *trpB*R.

RESULTS

Using BOX-PCR analysis (300 – 3000 bp) all type strains were successfully discriminated, producing different banding patterns (Table 1). In terms of the 150 *Streptomyces* isolates, 24 unique fingerprints were derived, defining the isolates as 24 different strains. The phylogenetic relatedness of these isolates is presented in Table 2.

The specificity of the *trpB* primers designed was analysed by PCR. Products of the appropriate size (504 bp) were formed with all of the 13 type *Actinobacteria* strains and the 150 *Streptomyces* isolates [17], (Fig. 1). Additionally, 10 non-actinobacteria strains were screened and none gave a PCR product (data not shown). Further sequencing analysis of the above PCR products followed by a BLAST search (www.ncbi.nih.gov), resulted in hits of *trpB* sequences from *Actinobacteria*, confirming the capability of using the primers designed in this work for analysis of the *trpB* gene within this group of bacteria.

16S rRNA gene sequences of type *Streptomyces* strains and isolates were analysed and compared with similarity values among 93 and 100 %.

Table 2. *Streptomyces* isolates investigated in this study.

Isolate[a]	16S rDNA GeneBank accession number	Sequence alignment		Closest phylogenetic sequence		Closest phylogenetic relative[d]	W.P.
		No of nucleotides[b]	% identity[c]				
GRE8	DQ192111	870	100	*Streptomyces* DQ018284	sp.	*S. cyaneus*	0.956
GRE28	DQ192112	870	100	*Streptomyces* DQ018284	sp.	*S. cyaneus*	0.911
REA33	DQ192113	870	100	*Streptomyces* DQ018284	sp.	*S. cyaneus*	0.941
D118	DQ192114	870	100	*Streptomyces* DQ018284	sp.	*S. cyaneus*	0.972
D110	DQ192119	870	100	*Streptomyces* EU384283	sp.	*S. cyaneus*	0.952
ASE20	DQ192122	869	100	*Streptomyces* EU410509	sp.	*S. cyaneus*	0.933
D316	DQ192115	870	100	*Streptomyces* DQ018284	sp.	*S. albidoflavus*	0.944
AMO70	DQ192120	870	100	*Streptomyces* EU384283	sp.	*S. albidoflavus*	0.974
S151	DQ192134	870	100	*Streptomyces* AF128874	sp.	*S. albidoflavus*	0.912
D116	DQ192123	870	100	*Streptomyces* AF128874	sp	*S. albidoflavus*	0.912
D126	DQ192116	870	100	*Streptomyces* DQ018284	sp.	*S. albidoflavus*	0.954
D313	DQ192117	870	100	*Streptomyces* DQ018284	sp.	*S. albidoflavus*	0.922
D314	DQ192124	870	99.4	*S. ciscaucasicus* AB184208		*S. albidoflavus*	0.955

Table 2 continued..

D37	DQ192125	870	100	*S. finlayi* EU285476		*S.albidoflavus*	0.937
D111	DQ192126	870	100	*Streptomyces* AF128874	sp.	*S. exfoliatus*	0.926
GRE23	DQ192118	870	100	*Streptomyces* DQ018284	sp.	*S. exfoliatus*	0.924
REA75	DQ192127	870	100	*Streptomyces* AF128874	sp.	*S. exfoliatus*	0.944
KAS11	DQ192128	870	99.9	*S. globisporus globisporus* DQ026634	subsp.	*S. exfoliatus*	0.911
GRE18	DQ192121	870	100	*Streptomyces* EU384283	sp.	*S. exfoliatus*	0.986
REA17	DQ192129	868	99.9	*Streptomyces* EU054360	sp.	*S. exfoliatus*	0.957
S272	DQ192130	870	100	*Streptomyces* AF128874	sp.	*S. exfoliatus*	0.971
OL19	DQ192131	870	100	*Streptomyces avermitilis* BA000030		*S. rochei*	0.925
OL27	DQ192132	982	100	*Streptomyces* EU214956	sp.	*S. rochei*	0.988
OL28	DQ192133	919	99.5	*Streptomyces longisporoflavus* AB184220		*S. rochei*	0.937

[a]Origins of isolation: a) rhizosphere of evergreen woody srubs growing on secludes Aegean islands (strains AMO, OL) b) rhizosphere of endemic plant *Ebenus sibthorpii* (strains ASE, D, REA) c) rhizosphere of indigenous *Pinus brutea* from the island of Crete (strains GRE) d) rhizosphere of evergreen woody shrubs growing on secludes Ionian islands (strains S, KAS) (Katsifas, *et al.*, 1999). [b]The number of 16S rDNA nucleotides used for the alignment. [c]The percentage identity with the 16S rDNA sequence of the nearest phylogenetic neighbour. [d]Based on 41 morphological and physiological diagnostic characters (Williams, *et al.*, 1983) and assessed for reliability of identification with Wilcox probability (W.P.).

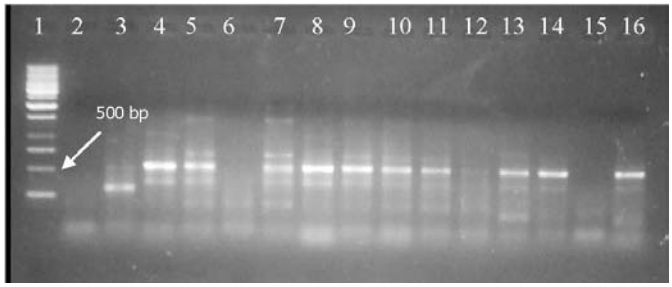

Figure 1. Example of PCR using primers trpBF/R: (1) 1kb DNA ladder GENE RULER (FERMENTAS®), (2) negative control (3) *Agrobacterium tumefaciens* DSM5172, (4) *Streptomyces aureofaciens* DSM40127, (5) *Streptomyces lydicus* DSM40461, (6) *E. coli* DSM8830, (7) *Saccharothrix syringae* DSM43886, (8) *Nocardia veterana* DSM40777, (9) *Actinosynema mirum* DSM43827, (10) isolate GRE28, (11) isolate S151, (12) isolate AMO70, (13) isolate ASE20, (14) isolate REA17, (15) *Pseudomonas putida* DSM4476 (negative control), and (16) *C. glutamicum* ATCC21253 (positive control).

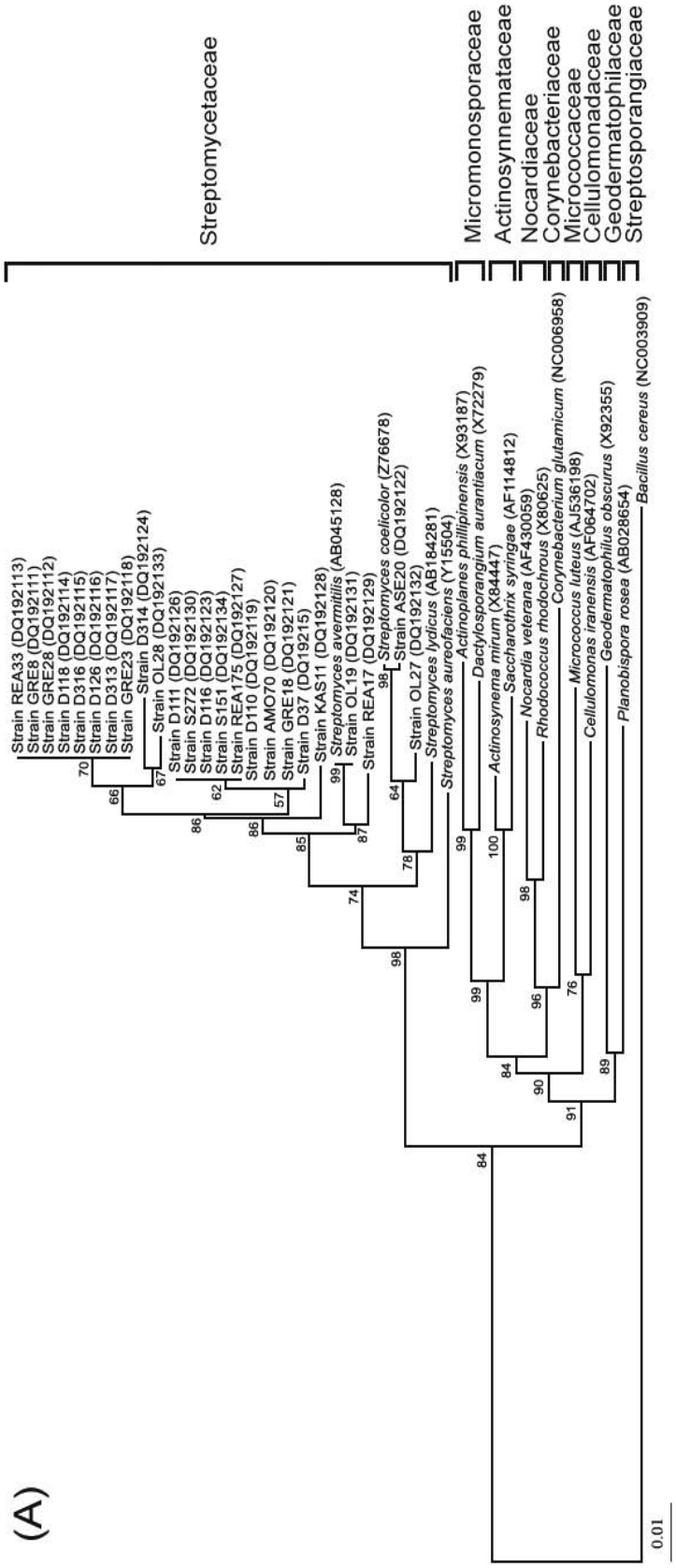
(A)
Streptomycetaceae
Micromonosporaceae
Actinosynnemataceae
Nocardiaceae
Corynebacteriaceae
Micrococcaceae
Cellulomonadaceae
Geodermatophilaceae
Streptosporangiaceae
Strain REA33 (DQ192113)
Strain GRE8 (DQ192111)
Strain GRE28 (DQ192112)
Strain D118 (DQ192114)
Strain D316 (DQ192115)
Strain D126 (DQ192116)
Strain D313 (DQ192117)
Strain GRE23 (DQ192118)
Strain D314 (DQ192124)
Strain OL28 (DQ192133)
Strain D111 (DQ192126)
Strain S272 (DQ192130)
Strain D116 (DQ192123)
Strain S151 (DQ192134)
Strain REA175 (DQ192127)
Strain D110 (DQ192119)
Strain AMO70 (DQ192120)
Strain GRE18 (DQ192121)
Strain D37 (DQ19215)
Strain KAS11 (DQ192128)
Streptomyces avermitilis (AB045128)
Strain OL19 (DQ192131)
Strain REA17 (DQ192129)
Streptomyces coelicolor (Z76678)
Strain ASE20 (DQ192122)
Strain OL27 (DQ192132)
Streptomyces lydicus (AB184281)
Streptomyces aureofaciens (Y15504)
Actinoplanes phillipinensis (X93187)
Dactylosporangium aurantiacum (X72279)
Actinosynema mirum (X84447)
Saccharothrix syringae (AF114812)
Nocardia veterana (AF430059)
Rhodococcus rhodochrous (X80625)
Corynebacterium glutamicum (NC006958)
Micrococcus luteus (AJ536198)
Cellulomonas iranensis (AF064702)
Geodermatophilus obscurus (X92355)
Planobispora rosea (AB028654)
Bacillus cereus (NC003909)
0.01

Figure 2

Figure 2 continued..

(B)

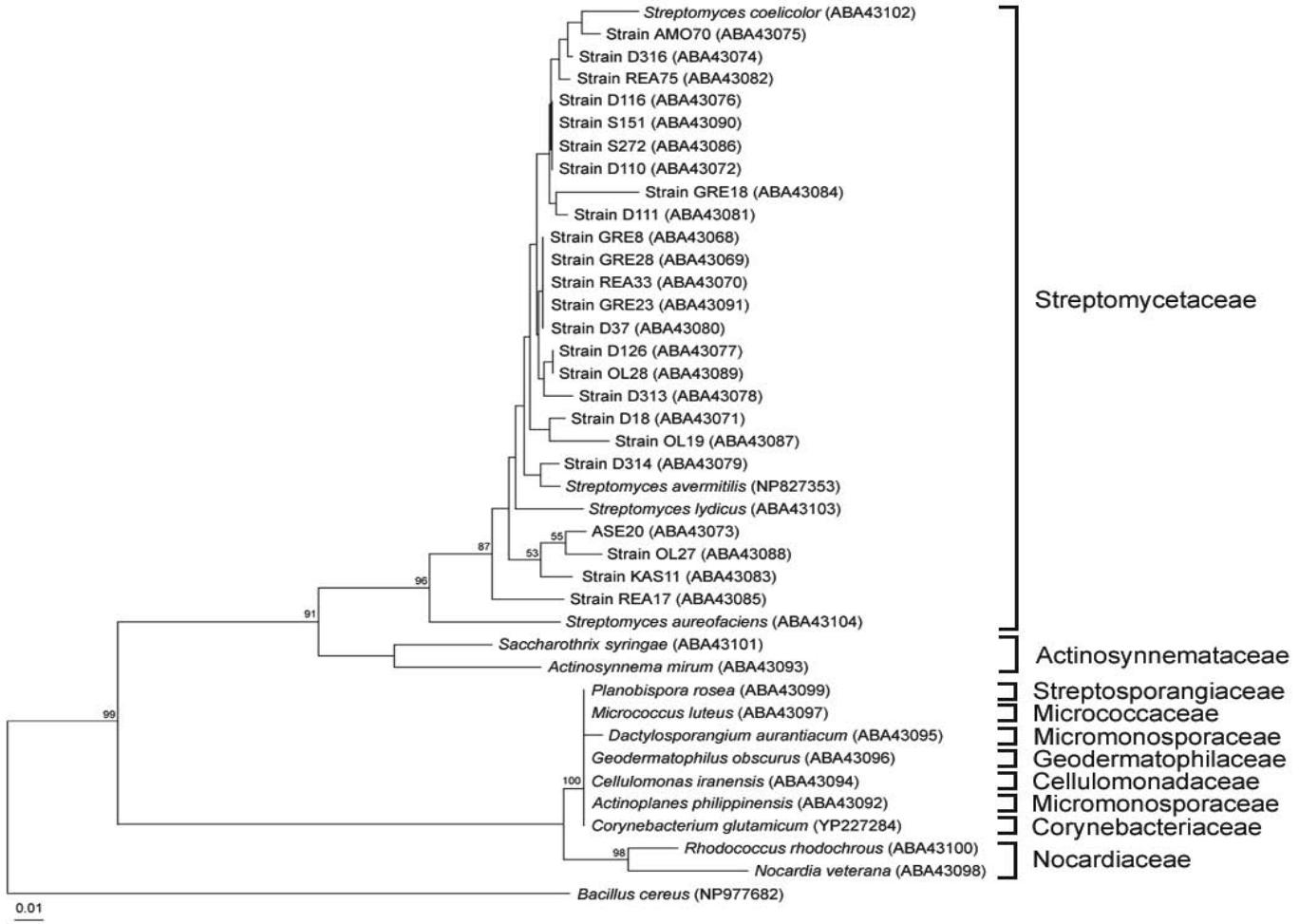

Figure 2. Phylogenetic trees based on 16S rRNA (ca. 1500 bp) (A) and deduced amino acid sequences of *trpB* genes (ca. 500 bp) (B) of the *Actinobacteria* strains used in this work, based on the neighbour-joining method as determined by distance Jukes-Cantor analysis. One thousand bootstrap analyses (distance) were conducted, and percentages greater than 50 % are indicated at the nodes. The tree was rooted with Bacillus cereus. Scale bar represents 1 % estimated distance.

trpB gene fragments were amplified from both type strains and isolates and the nucleotide sequences were determined and compared. A *trpB* database for *Streptomyces* species was then formed. The strains used in this study showed from 86 to 100 % similarity of *trpB* sequences analysed, allowing a more accurate discrimination of the species. In particular, 16S rRNA and *trpB* sequence comparisons were performed, confirming more than 98 % similarity for the 65.5 % of the 16S rRNA sequences compared, while the percentage of *trpB* sequences with more than 98 % similarity was only 7.7 %. Furthermore, the percentage of sequences with identity higher than 95 % was 98.6 % for 16S rRNA and 16.2 % for *trpB*.

Based on the phylogenetic relationships of the 16S rRNA and deduced amino acid sequences from partial *trpB* sequences, the studied *Streptomyces* strains were grouped in similar branches (Fig. 2). DGGE analysis of actinobacteria community in natural environments, which exhibit a high degree of streptomycete diversity, led to similar estimation of diversity, whether using 16S *Actinobacteria* group specific primers or the *trpB* novel primers, as estimated by the number of amplified bands revealed (Fig. 3).

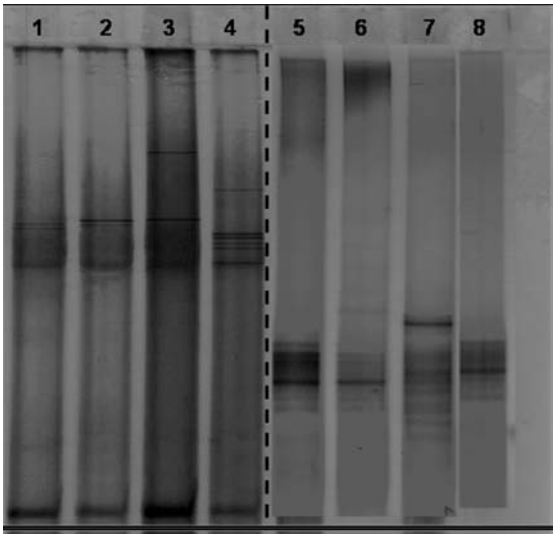

Figure 3. DGGE analysis of total actinobacteria community based on trpB sequence (1-4) and 16S rRNA (5-8). Lanes 1, 5: rhizosphere of evergreen woody srubs growing on secludes Aegean islands, Lanes 2, 6: rhizosphere of endemic plant Ebenus sibthorpii, Lanes 3, 7: rhizosphere of indigenous Pinus brutea from the island of Crete, Lanes 4, 8: rhizosphere of evergreen woody shrubs growing on secludes Ionian islands.

DISCUSSION

In this study, we investigated the phylogenetic divergence within the group of actinobacteria, providing an additional target gene, also capable of demonstrating community diversity within actinobacteria colonizing diverse environments. The 24 unique fingerprints were represented by only 14 different (with homology from 95 to 99 %) 16S rRNA sequences, implying that by using 16S rRNA sequencing underestimation of diversity at species level can occur. The discriminatory power of BOX-PCR for distinguishing between related strains of the same species has been shown generally in several bacterial groups [25], as well as within the *Actinobacteria* [26, 27, 28, 29]. However, the poor correlation between number of strains as estimated by BOX-PCR and 16S rRNA sequencing has been attributed to the fact that 16S rRNA is a highly conserved gene [30, 31]. *Streptomyces* strains can undergo chromosomal rearrangements such as deletions and amplifications, that directly affect BOX fingerprint whereas 16S rRNA sequence remain conserved [32, 27].

In terms of the 16S rRNA gene sequences of type *Streptomyces* strains and isolates, similarity values between 93 and 100 % were retrieved, in agreement with Hain *et al.* [10] who investigated the use of 16S rRNA probes to determine intraspecific relationships within *S. albidoflavus*. Despite the general use of the 16S rRNA gene as framework for modern bacterial classification, it has often been proved to show limited variation for the discrimination of closely related taxa and strains [33]. On the other hand, protein coding genes exhibit higher genetic variation, which can be used for the classification of closely related species [34, 35, 36, 37, 16]. Protein coding and housekeeping genes exhibit much higher genetic variation than 16S rRNA. Such genes have been widely used during the last years for classification of closely related taxa in actinobacteria as well as in other Gram positive groups [38, 3, 14, 39, 34].

Sequence analysis of the *trpB* gene demonstrated a range of similarity between 86 to 100 %, thus

allowing a more accurate discrimination of the species. Sequence similarities of the *trpB* gene could be used to distinguish between closely related strains such as GRE8, GRE23, GRE 28, REA33, D118, D126, D313 and D316, which showed 100 % sequence similarity for the 16S rRNA gene. However, the sequence similarities of the above strains for *trpB* gene ranged from 90 to 99 %. Only GRE8, GRE23 and GRE28 isolates could not be distinguished with the use of the *trpB* gene sequence, by exhibiting 100 % sequence similarity with each other. Furthermore, *trpB* gene sequences for isolates S151, S272, REA75, D111 and D116 exhibited similarity values among 97 and 99 %, while the sequence similarity for the 16S rRNA gene was also 100 %. Isolates GRE18, AMO70 and D110 also exhibited 100 % sequence similarity for the 16S rRNA gene. However, the sequence similarities of GRE18 - D110, GRE18 - AMO70 and AMO70 - D110 for *trpB* gene were 92 %, 91 % and 97 % respectively for each one of the pairs. Additionally, in other studies [14] *trpB* has also been used successfully as a genotyping approach to overcome obstacles from 16S rRNA gene similarity in phylogenetic studies within *Streptomyces*. The different region of *trpB* analysed by Egan *et al.* [14] was useful in clarifying relationships between *S. griseus* and *S. humidus* isolates.

Molecular phylogeny based on the 16S rRNA gene revealed a distinct Streptomycetaceae clade encompassing all the examined *Streptomyces* strains. Based on *trpB*, the same clade was observed, with similarly satisfactory bootstrap support. However, no phylogenetic distinction was feasible for the corresponding representatives of the rest of the investigated actinobacteria families, which were found to have highly similar phylogenies. This renders *trpB* sequence analysis an appropriate molecular tool for the identification of streptomycete strains only.

DGGE analysis of actinobacteria community in the selected environments based on 16S rRNA using actinobacteria specific primers revealed considerable diversity as estimated by the number of amplified 16S rRNA bands (Fig. 3). A similar pattern, comprising of equal number of phylotypes, occurred using the *trpB* primers for DGGE analysis. The *Actinobacteria* population mainly consisted of *Streptomyces* species found at high density demonstrated and supported the parallel use of *trpB* group specific primers, as a biodiversity estimation tool for natural streptomycete communities. The amplified region of the *trpB* gene was one of the longest described for *Actinobacteria* in literature [16], providing useful information and an alternative streptomycete diversity marker for complementary resolution in molecular environmental techniques, such as DGGE analysis [14].

In conclusion, our results suggested that *trpB* can be used as an alternative molecular marker to 16S rRNA gene for successfully resolving phylogenies between closely related species and subspecies within the genus *Streptomyces*. This study could also provide a framework for analysis of natural streptomycete communities and for the exploitation of the use of *trpB* gene as a *Streptomyces* diversity estimation tool.

ACKNOWLEDGMENTS

The work was supported by the European Commission (project ACTAPHARM, 5th framework, QLK3-CT-2001-01783).

REFERENCES

1. McCarthy, A. J. 1987, FEMS Microbiol. Rev., 46, 145.
2. Tanaka, Y., and Omura, S. 1990, Actinomycetologica, 4, 13.
3. Anderson, A. S., and Wellington, E. M. 2001, Int. J. Syst. Evol. Microbiol., 51, 797.
4. Stackebrandt, E., Witt, D., Kemmerling, C., Kroppenstedt, R., and Liesack, W. 1991, Appl. Environ. Microbiol., 57, 1468.
5. Kataoka, M., Ueda, K., Kudo, T., Seki, T., and Yoshida, T. 1997, FEMS Microbiol. Lett., 151, 249.
6. Piza, F. F., Prado, P. I., and Manfio, G. P. 2004, Antonie Van Leeuwenhoek, 86, 317.
7. Eisen, J. A. 1995, J. Mol. Evol., 41, 1105.
8. Hirsch, A. M., McKhann, H. I., Reddy, A., Liao, J., Fang, Y., and Marshall, C. R. 1995, Mol. Biol. Evol., 12, 16.
9. Koch, C., Kroppenstedt, R. M., Rainey, F. A., and Stackebrandt, E. 1996, Int. J. Syst. Bacteriol., 46, 765.

10. Hain, T., Ward-Rainey, N., Kroppenstedt, R. M., Stackebrandt, E., and Rainey, F. A. 1997, Int. J. Syst. Bacteriol., 47, 202.
11. Heuer, H., Krsek, M., Baker, P., Smalla, K., and Wellington, E. M. 1997, Appl. Environ. Microbiol., 63, 3233.
12. Wang, Y., Zhang, Z., and Ramanan, N. 1997, J. Bacteriol., 179, 3270.
13. Liu, S. L., and Sanderson, K. E. 1998, FEMS Microbiol. Lett., 164, 275.
14. Egan, S., Wiener, P., Kallifidas, D., and Wellington, E. M. 2001, Antonie Van Leeuwenhoek, 79, 127.
15. Huddleston, A. S., Cresswell, N., Neves, M. C., Beringer, J. E., Baumberg, S., Thomas, D. I., and Wellington, E. M. 1997, Appl. Environ. Microbiol., 63, 1288.
16. Guo, Y., Zheng, W., Rong, X., and Huang, Y. 2008, Int. J. Syst. Evol. Microbiol., 58, 149.
17. Katsifas, E. A., Giannoutsou, E. P., and Karagouni, A. D. 1999, Lett. Appl. Microbiol., 29, 48.
18. Williams, S. T., Goodfellow, M., Wellington, E. M., Vickers, J. C., Alderson, G., Sneath, P. H., Sackin, M. J., and Mortimer, A. M. 1983, J. Gen. Microbiol., 129, 1815.
19. Hopwood, D. A., Bibb, M. J., Chater, K. F., Kieser, T., Bruton, C. J., Kieser, H. M., Lydiate, D. J., Smith, C. P., Ward, J. M., and Schrempf, H. 1985, Genetic Manipulation of *Streptomyces*: a Laboratory Manual, The John Innes Foundation, Norwich.
20. Versalovic, J., Schneider, M., de Bruijn, F. J., and Lupski, J. R. 1994, Meth. Cell Mol. Biol., 5, 25.
21. Rademaker, J. L. W., and de Bruijn, F. J. 1997, Characterizaton and Classification of Microbes by rep-PCR Genomic Fingerprinting and Computer-assisted Pattern Analysis. In: DNA Markers: Protocols, Applications and Overviews, Caetano-Anollés G., and Gresshoff, P. M. (Eds.), J. Wiley & Sons Inc., New York, 151.
22. Lane, D. J. 1991, Nucleic Acid Techniques in Bacterial Systematics, Stackebrandt, E., and Goodfellow, M. (Eds.), Wiley, New York, 115.
23. Swofford, D. L. 2003, PAUP* Phylogenetic Analysis Using Parsimony (*and Other Methods) Version 4, Sinauer Associates, Sunderland, Massachusetts.
24. Riesner, D., Steger, G., Zimmat, R., Owens, R. A., Wagenhofer, M., Hillen, W., Vollbach, S., and Henco, K. 1989, Electrophoresis, 10, 377.
25. Versalovic, J., Koeuth, T., and Lupski, J. R. 1991, Nucleic Acids Res., 19, 6823.
26. Alves, A., Santos, O., Henriques, I., and Correia, A. 2002, FEMS Microbiol. Lett., 6, 205.
27. Davelos, A. L., Xiao, K., Samac, D. A., Martin, A. P., and Kinkel, L. L. 2004, Microb. Ecol., 48, 601.
28. de Oliveira, E. J., Rabinovitch, L., Monnerat, R. G., Passos, L. K., and Zahner, V. 2004, Appl. Environ. Microbiol., 70, 6657.
29. Huang, Y., Li, W., Wang, L., Lanoot, B., Vancanneyt, M., Rodriguez, C., Liu, Z., Swings, J., and Goodfellow, M. 2004, Int. J. Syst. Evol. Microbiol., 54, 2085.
30. Dharmalingam, K., and Cullum, J. 1996, J. Biosciences, 21, 433.
31. Fischer, G., Kyriacou, A., Decaris, B., and Leblond, P. 1997, Biochimie, 79, 555.
32. Leblond, P., and Decaris, B. 1994, FEMS Microbiol. Lett., 123, 225.
33. Nübel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R. I., Ludwig, W., and Backhaus, H. 1996, J. Bacteriol., 178, 5636.
34. Ko, K. S., Kim, J. M., Kim, J. W., Jung, B. Y., Kim, W., Kim, I. J., and Kook, Y. H. 2003, J. Clin. Microbiol., 41, 2908.
35. Bavykin, S. G., Lysov, Y. P., Zakhariev, V., Kelly, J. J., Jackman, J., Stahl, D. A., Cherni, A. 2004, J. Clin. Microbiol., 42, 3711.
36. Konstantinidis, K. T., and Tiedje, J. M. 2005, J. Bacteriol., 187, 6258.
37. Zeigler, D. R. 2005, Int. J. Syst. Evol. Microbiol., 55, 1171.
38. Wiener, P., Egan, S., Huddleston, A. S., and Wellington, E. M. 1998, Mol. Ecol., 7, 1205.
39. De Clerck, E., and De Vos, P. 2004, FEMS Microbiol. Lett., 9, 91.