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Investigation of actinomycete diversity in the tropical rainforests of Singapore

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Five thousand actinomycetes were isolated from soil samples collected from rainforests in Singapore and the generic identities of these isolates were determined by using a procedure that combined morphological, chemotaxonomic and 16S rDNA sequence-based phylogenetic analyses. Actinomycetes belonging to a total of 36 genera were identified. The most abundant isolates are members of *Streptomyces*, *Micromonospora*, *Actinoplanes*, *Actinomadura*, *Nonomuria*, *Nocardia* and *Streptosporangium*. By phylogenetic analysis of 16S rDNA sequences of our isolates together with those of known actinomycete species, we also evaluated the species diversity of several genera including *Streptomyces*, *Micromonospora*, *Nonomuria*, and *Actinomadura*. We found that: first, the tropical isolates are present in most clades represented by known species; and second, many tropical isolates form new clades distant from the known species, indicating the presence of unidentified taxa at both species and genus levels. Based on these results, we conclude that actinomycete diversity in the tropical rainforest is very great and should represent an excellent source for discovery of novel bioactive compounds.

Keywords: actinomycete diversity; phylogeny; rRNA; tropical rainforest

Introduction

Actinomycetes [4,28,29] have been especially useful to the pharmaceutical industry for their seemingly unlimited capacity to produce secondary metabolites with diverse chemical structures and biological activities. Tens of thousands of such compounds have been isolated and characterized, many of which have been developed into drugs for treatment of a wide range of human diseases [5,8]. Searching for novel actinomycetes constitutes an essential component in natural product-based drug discovery.

Actinomycetes are widely distributed in soil, water and other natural environments. However, the population and types of actinomycetes in an ecosystem are determined by numerous physical, chemical and biological factors. Identification of novel ecological systems is therefore crucial for the discovery of novel actinomycetes. Equatorial Southeast Asia is well known for its species-rich tropical rainforests representing ‘hotspots’ with rich biodiversity [5,20]. Located slightly north of the equator, Singapore has a warm and wet climate throughout the year. Some areas of Singapore are covered by tropical rainforest harboring numerous species of plants and animals. Though the plant and animal species have been reasonably well documented, information about microbial diversity in Singapore and Southeast Asia remains scarce. The biodiversity inventory of Singapore published in 1994 contains a list of 8000 species of organisms [30,31]. Surprisingly, only a few of those reported were bacteria. In order to gain insight into the microbial diversity in the rainforests of Singapore and Southeast Asia, in 1993 we initiated our microbial diversity study by first exploring the diversity of actinomycetes because of their enormous potential as a novel source for the discovery of new bioactive compounds. Our primary goals were twofold: (1) to determine the number of actinomycete genera from which representatives can be isolated in the rainforests; and (2) to acquire data that would allow us to evaluate the actinomycete diversity at species level.

Morphological features together with the composition of cell wall diaminopimelic acids (DAPs) and whole cell sugars are sufficient for accurate identification of actinomycetes of many genera [12,18], These properties can be determined quickly by using simple techniques such as light microscopy and thin-layer-chromatography. However, this approach may fail to correctly identify species of several genera that exhibit similar morphological and chemotaxonomic properties, such as members of *Nonomuria*, *Microbispora*, *Microtetraspora* and *Actinomadura* [16,33,38].

16S rRNA sequence-based phylogenetic analysis has been widely used to determine taxonomic positions of many organisms in virtually all taxonomic ranks [7,21,29,36]. Nowadays, many bacteria including ones which cannot be cultured in the laboratory, are identified solely by analyzing their 16S rRNA or gene sequences (rDNA) [1]. Assisted by the PCR technique and the ability to directly sequence PCR products, the 16S rDNA sequence of an unknown organism can be quickly obtained and immediately compared with thousands of sequences in public databases. However, DNA sequence analysis is rather expensive when thousands of sequences need to be determined.

In this study, we combined the above two approaches in a complementary way. Briefly, we examined 5000 acti-

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nomycete isolates by using the first approach. Isolates that could not be identified by this approach without ambiguity were then subjected to 16S rDNA sequence analysis. Here, we report the identification of actinomycetes belonging to 36 genera. By phylogenetic analysis using the 16S rDNA sequences from both the new isolates and known species, we also obtained evidence for high species diversity and existence of unidentified taxa of actinomycetes in the tropical rainforests of Singapore.

Materials and methods

Isolation of actinomycetes

A total of 100, 100, 80 and 70 soil samples were collected from Bukit Timah Nature Reserve (primary forest), Sungei Buloh (mangrove swamp forest), Sentosa Island (scattered primary and secondary forest), and Singapore Botanic Garden (patches of primary forest), respectively. For all samples, soil from the surface to about 10 cm deep was collected as a mixture.

Soil samples were processed on the day of collection. Each sample was first air-dried in a chemical fume hood for 3 days and then ground in a mortar. The sample was divided into two parts, one being heated at 80°C and the other at 120°C for 60 min. Five grams of each sample were then suspended in 20 ml sterile water and 100-μl aliquots of a serial dilution (1:10) were spread onto isolation plates whose surfaces had been dried in a laminar-flow hood. The following media were used for isolation plates: GYP agar, ISP2, ISP3, ISP4, Czapek's sucrose agar, Starch casein agar, Glucose asparagine agar, Malt yeast extract agar, Bennett's agar (recipes for the above media are described in Ref [2]), HU agar [10] and HV agar [10]. To minimize the growth of fungi and undesirable bacteria, nystatin (25 μg ml^{-1}), cycloheximide (50 μg ml^{-1}) and potassium dichromate (50 μg ml^{-1}) or nalidixic acid (50 μg ml^{-1}) were added to the isolation media [10].

To reduce the number of fast-growing non-actinomycetes, another pretreatment of the soil samples was to suspend 5 g of the air-dried soil in 20 ml LB medium supplemented with 10 μg ml^{-1} of ampicillin or penicillin and shake the suspension at 200 rpm at 37°C for 3 h before spreading aliquots of the serially-diluted suspension onto isolation plates.

To increase the possibility of isolating rare actinomycetes, various chemicals and antibiotics were added to different isolation plates, such as 0.05% SDS, 0.25% phenol, 15 μg ml^{-1} novobiocin, 25 μg ml^{-1} rifamycin, 25 μg ml^{-1} tunicamycin, 15 μg ml^{-1} streptomycin, 25 μg ml^{-1} kanamycin, 10 μg ml^{-1} miconazola, 0.8 μg ml^{-1} penicillin and 4 μg ml^{-1} polymycin B [10]. The chemotactic method described by Palleroni [22] was used for isolation of zoospore-producing actinomycetes.

The isolation plates were incubated at 28–30°C for 7–15 days for fast-growing actinomycetes or up to 35 days for slow-growing ones. Actinomycete colonies on the isolation plates were examined using a stereoscope (Leica Wild M8, magnification ×90) and picked on the basis of morphological features and colors of pigmentation including diffusible pigments. Selected colonies were transferred to ISP4 or Bennett's agar slants for further taxonomic analysis.

Identification of actinomycetes

Identification of actinomycetes to genus level was conducted by first using morphological and chemical criteria according to the guide described in Bergey's Manual of Determinative Bacteriology [12]. Isolates which could not be identified by their morphological and chemotaxonomic features were then identified by 16S rDNA sequence analysis.

For morphological observations, the actinomycetes were grown on glass cover slips which were half-way inserted into ISP4 agar plates as previously described [10]. The plates were incubated at 30°C for 1–2 weeks or longer for the slow-growing actinomycetes. Cells on the cover slips were examined using a light microscope for the description of morphological properties.

For chemotaxonomic studies, aerial and substrate mycelia of actinomycetes were scraped from the same plates used for morphological observations. The mycelia were processed and the isomers of diaminopimelic acids (*LL*-DAP or *meso*-DAP) and whole cell sugar patterns were determined as previously described [18,19]. Precoated cellulose plates (Merck, Darmstadt, Germany) were used for thin layer chromatograpy (TLC).

Preparation of genomic DNA

The preparation of genomic DNA of actinomycetes was carried out as described [33].

PCR amplification and sequencing of 16S rDNA

Two oligonucleotide primers that would amplify an approximately 900-bp fragment of rRNA gene were used in PCR reactions. The sequences of the two oligonucleotides are: 5′AGAGTTTGATCCTGGCTCAG 3′ targeting nucleotides (nt) 8–24 of *Escherichia coli* 16S rRNA [3] and 5′CCCCGTCAATTCATTTGA 3′ (nt 933–915). PCR amplification of 16S rDNA was carried out as described [33]. PCR products were resolved on a low-melting-point agarose gel, excised from the gel and purified using a QIAquick Spin PCR purification kit (Qiagen GmbH, Hilden, Germany). About 1 μg of a purified PCR product was sequenced from both ends using the same primers as those used for the PCR amplification on an ABI automatic sequencer (Perkin-Elmer, model 377; Norwalk, CT, USA). To assess the potential effect of rDNA heterogeneity on the phylogenetic analysis, the PCR products from 50 randomly selected isolates were also cloned as described [33] and three clones from each PCR product were sequenced.

Database search and phylogenetic analysis

Phylogenetic identity of an actinomycete was first determined by searching Genbank using the BLAST program and then confirmed by performing both multiple sequence alignment for visual examination of genus-specific nucleotide signatures and construction of phylogenetic trees. The multiple sequence alignment and tree construction programs in the DNASTAR software package (DNASTAR, Inc, Madison, WI, USA) were convenient and reliable tools for this purpose. For more robust phylogenetic analyses, we used the neighbor-joining method in the ClustalV package [11,26]. The stability of tree topology was evaluated by performing 1000 bootstrap replications using the BOOTSTRAP program contained in the same software package.

Results

Identification of actinomycete isolates

By using the morphological and chemotaxonomic properties alone, we were able to achieve generic identification of more than 80% of the isolates, which include several frequently occurring genera such as 3204 isolates of *Streptomyces*, 755 of *Micromonospora* and 206 of *Actinoplanes*. A fraction (~10%) of these isolates were also cross-examined by 16S rDNA sequence analysis. Except for one case where a *Nocardioides* isolate was mis-identified as *Streptomyces*, no discrepancy was found between the generic identities determined by the two approaches. The reliable generic identification of members of these large genera by the first approach greatly reduced the number of isolates that required the application of the more costly DNA sequence analysis. The rest of the isolates (a total of 835) were identified by 16S rDNA sequence analysis following the examination of their morphological and chemotaxonomic properties. Table 1 summarizes the results of identification. Actinomycetes belonging to 36 genera were found. Excluding all identical or nearly identical sequences, a total of 350 partial 16S rDNA sequences have been submitted to the GenBank.

Evaluation of species diversity

To evaluate the species diversity of the actinomycetes, we needed to establish the relationships between our isolates and known species. A broad range of methods has been used for similar purpose, which are largely based on three sources of information: genomic, phenotypic and chemical (for review, see Ref [9]). In principle, they aim to find the levels of similarities between different environmental isolates and between them and the known species. Close relatedness of different environmental isolates with all or most known species and formation of new centers of variation by new isolates would indicate high species diversity and the presence of new taxa. In this study, we used 16S rDNA sequence-based phylogenetic analysis to investigate the species diversity of actinomycetes. We first focused our study on four genera: *Streptomyces*, *Micromonospora*, *Actinomadura* and *Nonomuria* for two reasons. First, a large majority of antibiotics of actinomycete origin were isolated from members of these genera; second, 16S rDNA sequences of about 100 known *Streptomyces* and nearly all valid species of the latter three genera had been determined [16,17,25,33,35,38]. Since there were a large number of isolates belonging to *Streptomyces* and *Micromonospora*, we tried to reduce the number to a manageable level for 16S rDNA sequence analysis without sacrificing too much on diversity. By microscopic examination of morphological properties we selected about 10% of the isolates from each of the two genera. Excluding identical sequences, 110 and 47 rDNA sequences from *Streptomyces* and *Micromonospora*, respectively, were subjected to phylogenetic analyses. The results are shown in Figures 1 and 2. We also analyzed 38 and 23 sequences belonging to *Actinomadura* and *Nonomuria* (Figure 3). Two features of the distribution pattern of the new actinomycete isolates relative to the known species strongly indicate high species diversity and the presence of novel taxa. First, the new isolates are quite evenly distributed into clades represented by known species throughout the tree. Second, many clades exist consisting exclusively of the new isolates (clades denoted by horizontal arrows). The genus of *Streptomyces* embraces several hundreds of species but only about 100 sequences are currently available from the databases. Thus, we cannot rule out the possibility that the clades consisting solely of new isolates are closely related to the known species that are left out in this study. However, for the genera *Micromonospora*, *Actinomadura* and *Nonomuria*, nearly all the valid and many invalid species were included. Therefore, the clades consisting of new isolates are more likely to represent novel taxa. Many bacteria may contain heterogeneous copies of rRNA genes and the level of sequence dissimilarity may be as high as 6% [6,34]. To address whether rDNA heterogeneity contributed to the distribution of the new isolates on the trees, the PCR-amplified rDNA of many isolates was cloned and multiple clones were sequenced. Though low levels of difference (<1%) were occasionally observed

Table 1 Actinomycete genera found in the rainforests in Singapore

Genus	No. of isolates	No. of submitted sequences	GenBank accession No.
(1) *Actinocorallia*	1	1	AF131298
(2) *Actinomadura*	280	34	AF131299–AF131332
(3) *Actinoplanes*	206	18	AF131333–AF131350
(4) *Actinosynnema*	1	1	AF131351
(5) *Amycolatopsis*	1	1	AF131352
(6) *Arthrobacter*	1	1	AF131353
(7) *Catellatospora*	3	3	AF131354–AF131356
(8) *Cellulomonas*	1	1	AF131357
(9) *Couchioplanes*	1	1	AF131358
(10) *Dactylosporangium*	8	8	AF131359–AF131366
(11) *Geodermatophilus*	1	1	AF131367
(12) *Glycomyces*	2	2	AF131368–AF131369
(13) *Herbidospora*	1	1	AF131370
(14) *Intrasporangium*	1	1	AF131371
(15) *Kineospora*	2	2	AF131372–AF131373
(16) *Kitasatospora*	21	7	AF131374–AF131380
(17) *Microbispora*	5	5	AF131381–AF131385
(18) *Micromonospora*	755	47	AF131386–AF131432
(19) *Microtetraspora*	4	4	AF131433–AF131436
(20) *Nocardia*	12	12	AF131437–AF131448
(21) *Nocardiopsis*	3	3	AF131449–AF131451
(22) *Nonomuria*	390	25	AF131449–AF131477
(23) *Planomonospora*	1	1	AF131477
(24) *Planotetraspora*	1	1	AF131478
(25) *Promicromonospora*	1	1	AF131479
(26) *Pseudonocardia*	3	3	AF131480–AF131482
(27) *Rhodococcus*	2	2	AF131483–AF131484
(28) *Saccharopolyspora*	7	7	AF131485–AF131491
(29) *Saccharothrix*	2	2	AF131492–AF131493
(30) *Spirillospora*	3	3	AF131493–AF131496
(31) *Streptoalloteichus*	3	3	AF131497–AF131499
(32) *Streptomyces*	3204	110	AF131500–AF131609
(33) *Streptosporangium*	50	16	AF131610–AF131625
(34) *Tsukamurella*	3	3	AF131626–AF131628
(35) *Verrucosispora*	3	3	AF131629–AF131631
(36) *Kribbella*	4	4	AF131632–AF131635
(37) Potential novel genus in *Nocardioidaceae*	2	2	AF131636–AF131637
(38) Potential novel genus in *Streptosporangiaceae*	3	3	AF131638–AF131640
(39) Potential novel genus in *Thermonosporaceae*	7	7	AF131641–AF131647

between different clones from the same isolates, they always aggregated closely on the tree (results not shown). Recently, Ueda *et al* [32] reported that only 2.5% of the *Streptomyces* strains showed more than one base substitution between different copies of the 16S rRNA gene in a 120-bp fragment containing the highly variable α region. Taken together, we conclude that the effect of rRNA gene heterogeneity is minimal on the results of phylogenetic analysis presented here.

Evidence for the presence of novel genera

The phylogenetic relationships between all the genera belonging to three closely related families: *Thermomonosporaceae*, *Streptosporangiaceae* and *Nocardiopsaceae* have been thoroughly investigated [24,33,35,38]. The 16S rDNA sequences of nearly all valid species of these genera have been determined. Thus, the phylogenetic positions of the isolates belonging to these groups can be determined with a high degree of certainty. On the phylogenetic tree shown in Figure 3, two clades consisting solely of new isolates were consistently and distantly related to all the established genera of the three families. The branches leading to the two clades are deep on the tree and supported by high bootstrap values (998 and 936 respectively). The two clades are very likely to represent new genera, though other taxonomic data are needed to confirm the phylogenetic results. Another potential new genus was identified in the analysis of *Nocardioides*-related isolates (Figure 4). Two isolates, IM7744 and 7747, formed one clade distantly related with members of other genera of the family *Nocardioidaceae*. The closest phylogenetic neighbors of IM 7744 and 7747 are strains of *Kribbella*, a new genus recently proposed by Park *et al* [23]. The distance between the clade of IM 7744 and 7747 and the clade of *Kribbella* is significantly longer than that separating the two genera *Aeromicrobium* and *Nocardioides*, indicating the possibility of IM 7744 and IM 7747 as members of a new genus.

Discussion

In this report, we describe the characterization of actinomycetes isolated from the tropical rainforests in Singapore and the evaluation of their diversity at genus and species levels. By using a wide range of isolation media and sample-treatment methods and an identification strategy combining morphological and chemotaxonomic properties with 16S rDNA sequence-based phylogenetic analysis, we identified, from a total of 5000 actinomycete isolates, representatives belonging to 36 known genera of actinomycetes. The total number of actinomycete genera found is apparently much higher than that reported in similar studies conducted in other geographic regions. Recently, Xu *et al* [37] and Jiang and Xu [15] reported the identification of 29 genera of actinomycetes (six of which are synonyms of others, the actual number is therefore 23) in the whole Yunnan province of China, a vast area with complex physical geography and several climate zones. In another report of actinomycete diversity in south China, a total of 21 genera were found from a study of nearly 10 000 strains [13]. Singapore is only a tiny fraction of Yunnan in size (641 km^2 *vs* 436150 km^2), but the number of actinomycete genera identified in this study is significantly higher. The result may indicate that in tropical rainforests, actinomycete diversity is indeed very high as for plant and animal species. The evidence for high species diversity is corroborated by the fact that actinomycetes isolated in the tropical rainforests in Singapore are, in phylogenetic terms nearly as diverse as the known species that were collected from many different geographic regions on this planet over many decades. However, this does not exclude the possibility that the results may simply reflect the different procedures and strategies used for isolation and identification in different studies. For example, members of the genus *Nonomuria* presently can only be identified by 16S rDNA sequence analysis, which was not used in the other studies [13,15,37]. The types of actinomycetes we isolated from four different locations did not exhibit significant differences, which may be explained by the lack of drastically different ecological environments on the small island of Singapore.

The numbers of actinomycete isolates of different genera vary over a wide range, reflecting, to certain extent, the relative abundance of each genus in the tropical rainforests. It is also certain that isolation procedures and human biases in selecting colonies are factors affecting the relative frequency of different groups of actinomycetes among the isolates. Consistent with the results of many other studies, streptomycetes are the most abundant [13–15,37]. *Micromonospora* isolates were the second most frequently found actinomycetes especially on plates containing novobiocin, a condition well known for its selectivity for *Micromonospora* [10]. From soil samples that had been pre-grown for 3 h in LB medium supplemented with 10 $\mu g\ ml^{-1}$ ampicillin, considerably more actinomycete colonies were observed on ISP4 isolation plates and many members of *Actinomadura* and *Nonomuria* were isolated. This is likely due to killing of most fast-growing non-actinomycete bacteria by ampicillin, while most slow-growing actinomycetes and their spores are resistant to the drug. As shown in Table 1, the majority of the actinomycete isolates belong to five genera, whereas members of most genera were found only once or a few times. It is therefore difficult to establish which medium or treatment is particularly efficient in isolating members of a certain genus, except the method [22] designed for isolating zoospore-producing actinomycetes which led to the isolation of many members of *Actinoplanes*. We believe that the intrinsic high actinomycete diversity in the tropical rainforest, our usage of a wide range of isolation media and sample-treatment methods and the sensitive identification by 16S rDNA sequence analysis collectively contributed to our isolation and identification of actinomycetes belonging to 36 genera.

The discovery of many new phylogenetic clusters of actinomycetes in our study is not surprising. Many previous studies of microbial diversity concluded the presence of numerous uncharacterized bacteria in nature. For example, by using pyrolysis mass spectrometry, Sanglier *et al* [27] investigated the relationship between a large number of environmental isolates of *Micromonospora* and type strains of known species. They also found that many of the new isolates clustered far away from known species, suggesting the presence of novel taxa. Huddleston *et al* [14] also reported the isolation from soybean rhizosphere soil

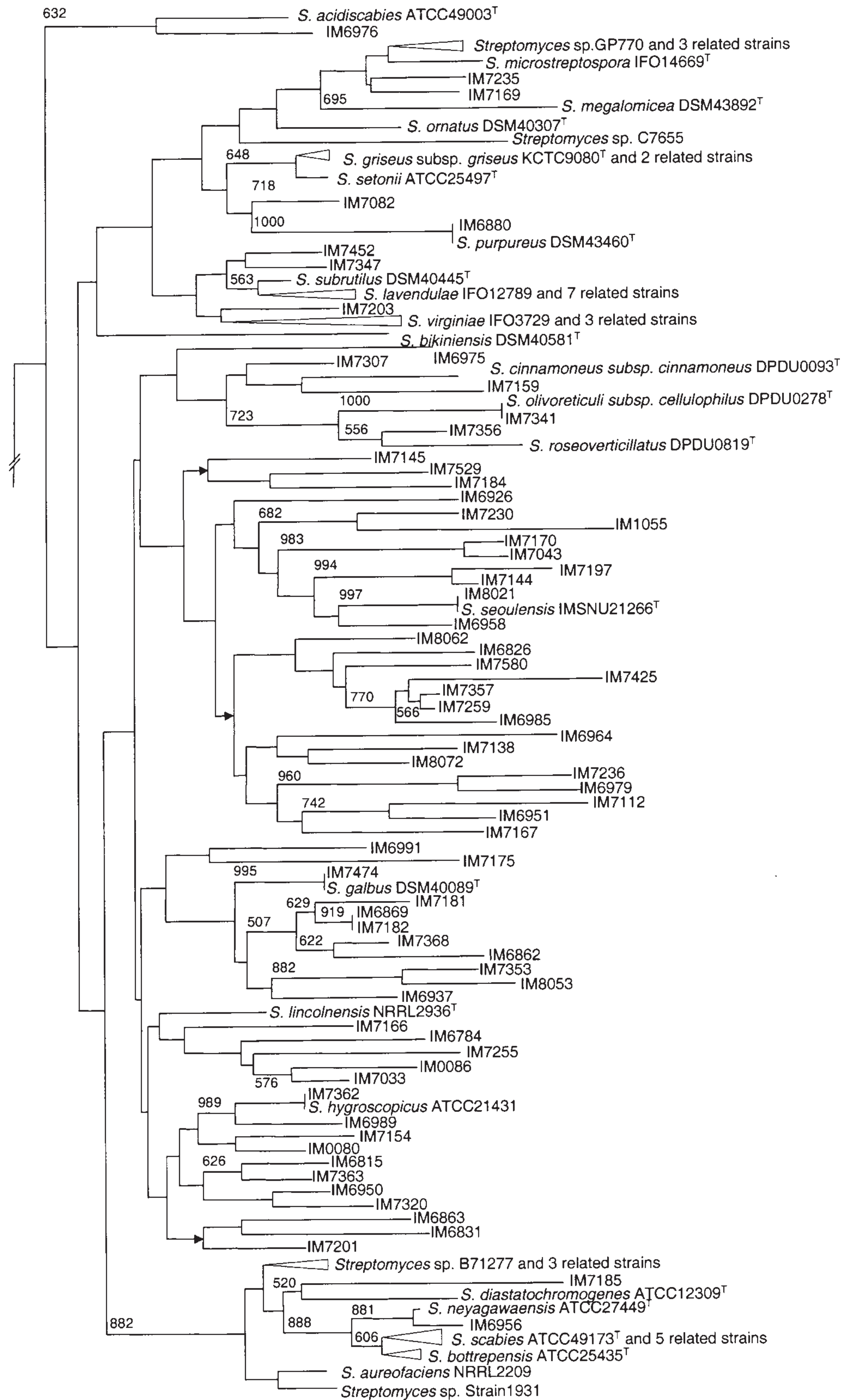

Figure 1 Phylogenetic tree of members belonging to the genus *Streptomyces*. The tree was reconstructed by using the neighbor-joining method [26]. The region from nucleotides 27–900 (*Escherichia coli* numbering [3]) of the 16S rDNA sequences was used for the analysis. The sequences of all the known actinomycete species were retrieved from the Genbank sequence database. The abbreviations for the culture collections are: ATCC, American Type Culture Collection, Rockville, MD, USA; DPDU, Istituto di Difesa delle Plante, Universita degli Studi di Udine, Udine, Italy; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; IFO, Institute of Fermentation, Osaka, Japan; JCM, Japan Collection of Microorganisms, Wako, Japan (*Continued*).

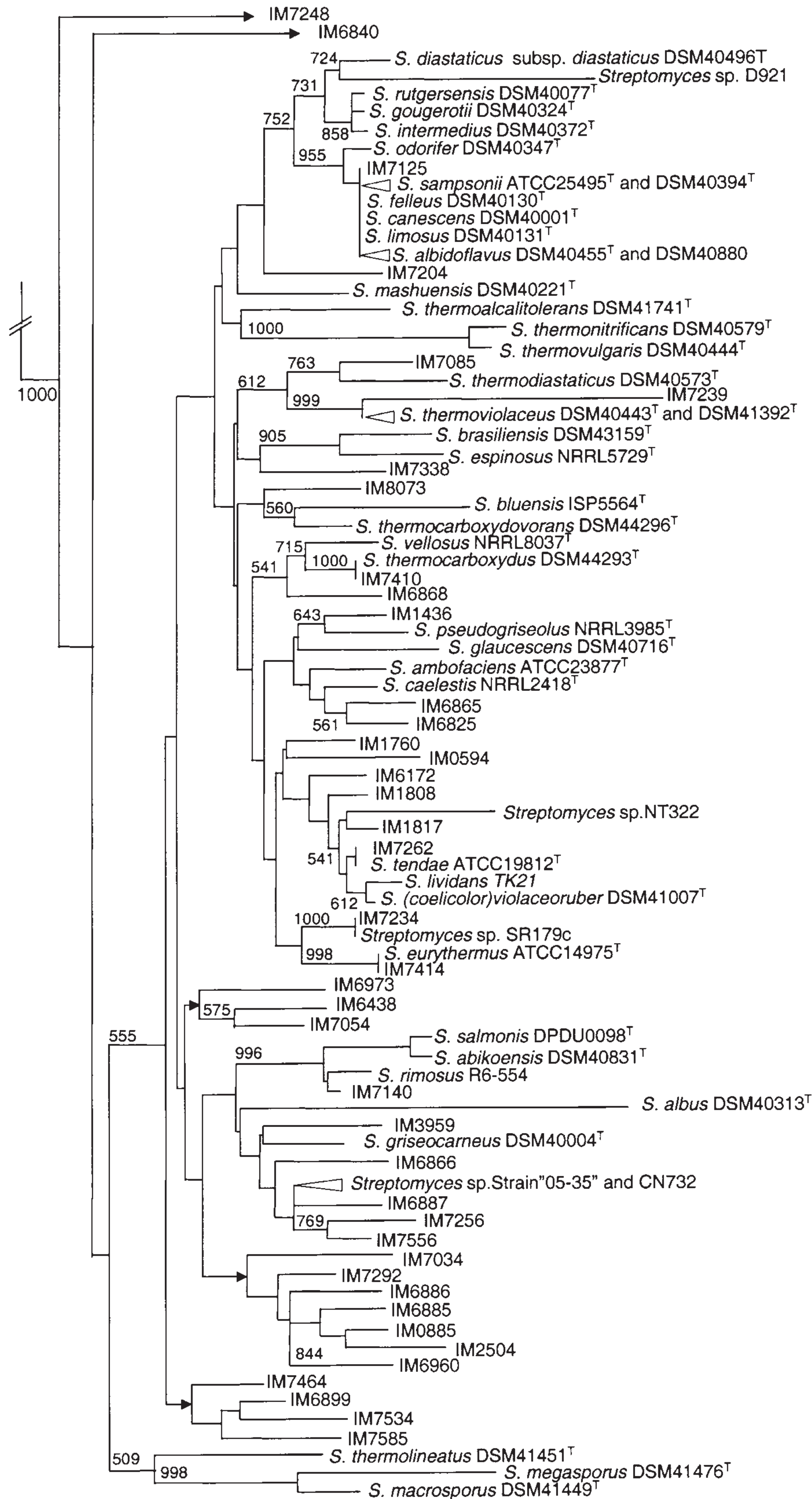

Figure 1 Continued. KCTC, Korean Collection of Type Cultures, Korean Research Institute of Bioscience and Biotechnology, Taejeon, Republic of Korea; NRRL, Northern Regional Research Laboratory, Agriculture Research Service, US Department of Agriculture, Peoria, IL, USA. The tree is presented in two parts which are connected by the vertical branches with an arrow at the end. The horizontal arrows point to the clades consisting solely of new isolates. The bar at the top indicates the number of inferred substitutions per 100 nucleotides. The numbers at the nodes indicate the levels of bootstrap support based on 1000 resamplings. This tree is part of a large tree constructed by including representative members of many other actinomycete genera.

Figure 2 The phylogenetic tree of isolates belonging to the genus *Micromonospora*. For details, see the legend to Figure 1. This tree is part of a tree constructed by including representative members of all genera in the family *Micromonosporaceae*.

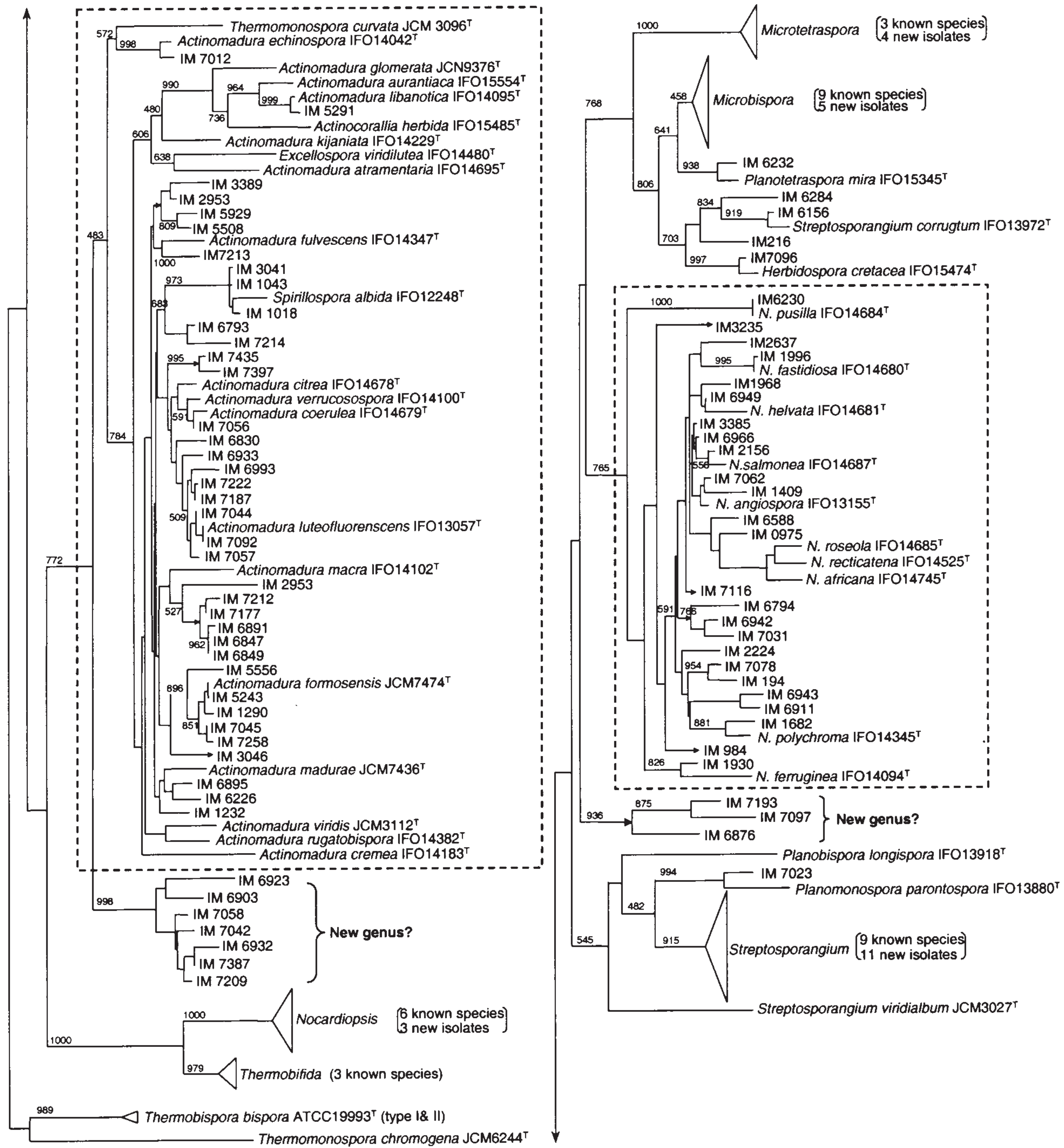

Figure 3 The phylogenetic tree of members belonging to the families *Thermomonosporaceae*, *Streptosporangiaceae* and *Nocardiopsaceae*. The two trees are continuous and connected by the vertical branches with an arrow at the end. For other details, see the legend to Figure 1. The dotted box on the left shows members of *Actinomadura* and several genera that intermix with *Actinomadura* species. The dotted box on the right shows members of *Nonomuria*.

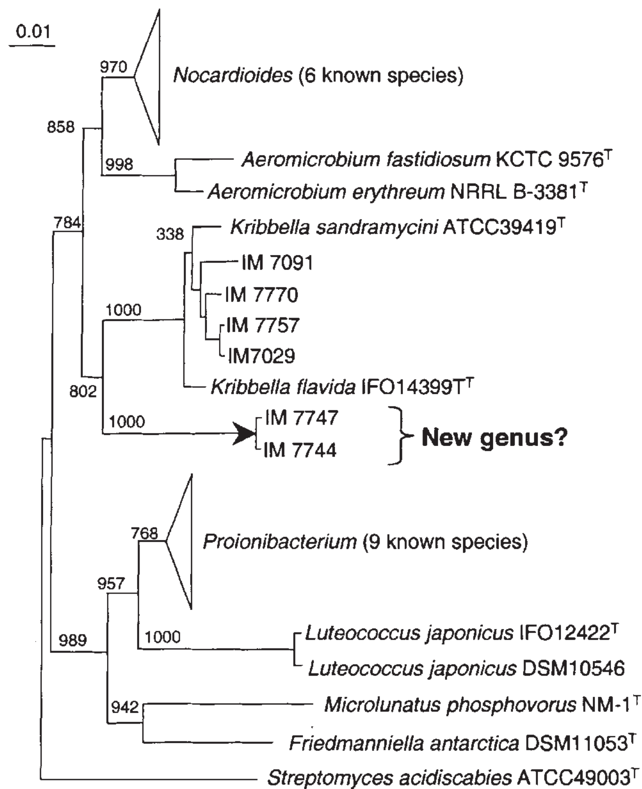

Figure 4 The phylogenetic tree of members belonging to the family *Nocardioidaceae*. For details, see the legend to Figure 1.

collected in Brazil of streptomycin-producing *Streptomyces* strains which could not be matched to existing species. By analyzing the 16S rDNA sequences PCR-amplified from environmental DNA, many researchers have reached the conclusion that the majority of microorganisms in nature have not been cultured in laboratories and remain uncharacterized (see review in Ref [1]).

On the basis of the results of this investigation, we conclude that the actinomycete diversity in the tropical rainforests in Singapore is high at both genus and subgenus levels. This result provides direct evidence for the view that tropical rainforests harbor high microbial diversity [5,20]. Since the ecosystem of the forests in Singapore is more or less representative of the vast areas of rainforests in Southeast Asian countries within the same tropical belt, the results should be valuable in estimating the actinomycete diversity in the whole region. So far, only a few isolated reports are available of systematic investigation of actinomycete diversity in a large geographic region and these reports provide little information about the diversity at subgenus level [13,15,37]. Thus, the results of this study and the large number of 16S rDNA sequences obtained from diverse actinomycetes may serve as a reference for similar studies in the future. For the pharmaceutical industry, the enormous and largely untapped actinomycete diversity in the tropical rainforests of equatorial Southeast Asia offers an excellent source for the discovery of novel bioactive compounds with therapeutic value.

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