A Comparative Study of Malaysian and Japanese Actinomycetes Using a Simple Identification Method Based on Partial 16S rDNA Sequence

Hideyuki Muramatsu^{1*}, Neelam Shahab², Yasuhisa Tsurumi¹ and Motohiro Hino¹

¹Exploratory Research Laboratories, Fujisawa Pharmaceutical Co. Ltd., 5–2–3, Tokodai, Tsukuba, Ibaraki, 300–2698, Japan

²Environment & Bioprocess Technology Centre, SIRIM Berhad, 1, Persiaran Dato' Menteri, Section 2, P.O. Box 7035, 40911 Shah Alam, Selangor, Malaysia

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A comparative analysis was performed on the taxonomy of actinomycete isolates of Malaysia and Japan. The taxonomical positions of isolates were determined using a simple identification method based on 16S rDNA partial sequence homology. The 16S rDNA partial sequence determination was made by direct PCR and direct sequencing. Simple identification was attempted for each of the 1128 actinomycete isolates for each country. We succeeded in simple identification of 790 Malaysian and 981 Japanese isolates. Malaysian isolates belonged to 9 families, 23 genera, and 185 species, including one genus as yet undescribed. The Japanese isolates belonged to 9 families, 22 genera, and 207 species. There was little taxonomical overlap between Malaysian isolates and Japanese isolates. Only 14% of the species and only 50% of the genera occurred in both groups. These results indicate that a group of actinomycetes of greater taxonomic diversity can be obtained when gathered from both regions, rather than from one or the other. Among Malaysian isolates, two strains were found that apparently do not belong to any known genus. Phylogenetic analysis indicates that they belong to the suborder *Sreptosporangineae*. The existence of candidate strains of a novel genus suggests that Malaysian actinomycetes have not been thoroughly investigated and therefore has promising potential as a source for novel bioactive compounds.

INTRODUCTION

Actinomycetes are well-known as a good source of natural product-based drug discovery programs. However, in recent times, the rate of discovery of novel bioactive compounds originating from actinomycetes has slowed¹⁾. Nevertheless, we believe that expanding the diversity of isolates will lead to renewed discovery.

Tropical rainforests are generally regarded as treasuries of biodiversity. The diversity of the tropical flora has been reported to be many times greater than that of the temperate flora^{2, 3)}. However, few reports have addressed the diversity of tropical actinomycetes^{4–6)}. Wang *et al.* reported on actinomycete diversity in the tropical rainforests of Singapore, finding 36 genera after identifying 5,000 isolates⁴⁾. Moreover, they discovered representatives of apparently novel genera^{4, 7)}. However, no report to date has examined numerous isolates to determine possible differences in diversity between actinomycetes in the Temperate Zone and that in the Tropical Zone.

To date, for handling of numerous strains, bacterial taxonomic position has primarily been determined by morphological observation, sometimes augmented by chemotaxonomical method^{4–6)}. However, these methods have

weaknesses in both accuracy and objectivity. For bacterial taxonomy, phylogenetic analysis by 16S rDNA sequences has become the standard approach. However, for the treatment of many isolates, DNA sequence analysis is costly in terms of money, time and effort. Recently, Tsuchizaki *et al.* have reported that colony direct PCR may also be applied to actinomycetes⁸⁾. Independently, we have devised direct PCR method using a culture broth. Combining this direct PCR method and the direct sequencing method, we devised a 16S rDNA partial sequence-based simple identification system that enabled simple identification of thousands of isolates.

This is the first reported analysis comparing actinomycetes gathered from a tropical rainforest and that from the Temperate Zone, using isolates numbering on the order of thousands, and based on a simple identification system involving partial 16S rDNA sequencing.

MATERIALS AND METHODS

Soil Samples

Malaysian soil samples were collected at Bukit Lagong Forest Reserves, Rawang, Selangor. This area is located in the center of Peninsular Malaysia at 3° 14' N and 101° 38'

*Corresponding author. Phone: +29-847-8611. Fax: +29-847-8313. E-mail: hideyuki_muramatsu@po.fujisawa.co.jp

E. Mean daily temperatures range from 27 to 32 °C. Annual rainfall is between 2000 to 2900 mm⁹⁾. Japanese soil samples were collected at Okutama, Tokyo, located on the Pacific side of central Honshu at 35° 48' N and 139° 3' E. Mean daily temperatures range from 0.1 to 23.2 °C (1979–2002). Annual rainfall is between 1092 to 2356 mm (1979–2000) (this data was collected at Ogouchi in the middle of Okutama)¹⁰⁾. Soil samples of both areas were collected in natural forests or forested areas. Soil samples were stored at 8°C until actinomycete isolation. Actinomycete isolation was performed within two months of soil sampling.

Actinomycetes isolation

We employed three methods to expand the diversity of the actinomycete isolates:

- 1) Air-drying method: 0.2–0.5 g of soil was air-dried for one week at room temperature in tubes. Then 2 ml of sterilized 0.01% polyoxyethylene sorbitan monooleate (Nacalai Tesque, Inc., Kyoto, Japan) was added to each tube and mixed vigorously. This suspension was sonicated in a bath-type sonicator for 5 min. The suspension was diluted and spread onto isolation plates. This is a general isolation method for actinomycetes.
- 2) Centrifugation method: We employed modified Takeuchi's method¹¹⁾. Air-dried soil (0.2–0.5 g) was gently mixed with 2 ml of sterilized 0.01% polyoxyethylene sorbitan monooleate without using a mixer. After incubation at 30 °C for 1 hr, 1 ml of supernatant was transferred to a sterile sampling tube. Centrifugation was performed at 7,000 rpm (3,000 × g), for 20 min. The supernatant was then diluted and spread onto isolation plates. This method allows isolation of actinomycete strains having motile spores.
- 3) Chloramin T method: We employed modified Hayakawa's method¹²⁾. Soil suspension was made by a procedure identical to air-drying method. 0.15 ml of soil suspension was mixed with the same volume of 4% Chloramin T (dissolved in sterile water; Nacalai Tesque, Inc.) solution. After incubation at 30 °C for 30 min, the suspension was diluted and spread onto isolation plates. The target taxon of this method is the family *Streptosporangiaceae*.

For isolation plates, we employed HV agar¹³⁾ plates with minor modification using "HUMAS" (Aiaisi kabusiki-gaisya, Osaka, Japan) as humic acid; 5 g/l. We also employed HV agar plates supplemented with antibiotic agents, streptomycin sulfate (5 mg/l; Sigma, St. Louis, U.S.A.) or novobiocin sodium salt (10 mg/l; Sigma) or erythromycin (2 mg/l; Sigma).

The isolation plates were incubated at 30 °C for 14 to 30 days. Actinomycete colonies on isolation plates were transferred to 1/5 SY agar, while seeking to avoid the repeated selection of colonies of identical morphology from the same plate. 1/5 SY agar was prepared as follows: soluble starch; 2 g/l (Nacalai Tesque, Inc.), yeast extract; 0.4 g/l (Nacalai Tesque, Inc.), agar; 16 g/l (after adjustment of pH to 7.3 with 1 N NaOH; Hayashi Pure Chemical Industries Ltd., Osaka, Japan), after autoclaving supplemented with cycloheximide (40 mg/l; Nacalai Tesque, Inc.).

Isolates for simple identification

2713 actinomycete strains were isolated from 85 Malaysian soil samples; 3690 actinomycete strains were isolated from 219 Japanese soil samples. For simple identification, 1128 isolates each from Japan and Malaysia were selected without morphological observation. To suppress any taxonomical skewing from isolation methods, the proportions of strains isolated by three isolation methods were kept nearly identical for the two regions. In selected strains, 36% of Malaysian and 34% of Japanese strains were isolated by the air-drying, general isolation method for actinomycetes. 13% of Malaysian and 12% of Japanese isolates were obtained by the centrifugation, zoospore selective method. 51% of Malaysian and 54% of Japanese isolates were obtained by the Chloramin T, the family *Streptosporangiaceae* selective method (Table 1).

Simple identification method

Simple identification was carried out according to the following five steps:

1) Preparation of culture broth

Each isolate was incubated in a well of a 96-well format plate. 100 µl of 1/2 strength ISP-Medium 1 (Bacto – Tryptone Yeast Extract Broth Dehydrated; Difco

Table 1. Isolates for simple identification.

Isolation Method		Air Drying ^{*1}					Centrifugation ^{*2}			CT ^{*3}	Total
Isolation Medium		HV	SM ^{*4}	EM ^{*5}	NV ^{*6}	Total(%)	HV	NV ^{*6}	Total(%)	HV(%)	
Number of Isolates	Malaysia	237	53	100	16	406(36)	105	46	151(13)	571(51)	1128(100)
	Japan	244	36	93	10	383(34)	93	47	140(12)	605(54)	1128(100)

^{*1} non-selective

^{*2} zoospore selective

^{*3} Chloramin T: *Streptosporangiaceae* selective

^{*4} HV agar supplemented with Streptomycin (5 mg/l)

^{*5} HV agar supplemented with Erythromycin (2 mg/l)

^{*6} HV agar supplemented with Novobiocin (10 mg/l)

Laboratories, Detroit, Michigan, U.S.A.) with 0.5% glycine (Kanto Chemical Co., Inc., Tokyo, Japan) and 0.01% polyoxyethylene sorbitan monooleate was dispensed to each well. A very small piece of the colony on 1/5 SY agar was inoculated in each well with a sterile toothpick. Incubation was performed at 30°C for about 16 hr without shaking. After incubation, plates were stored at -80 °C until direct PCR amplification.

2) PCR amplification and sequencing

Nested PCR and direct PCR methods were employed. The PCR primers were designed to be actinomycete-specific in order to suppress the influence of contamination. The primers of the first PCR were SRR181F (5'- GTT TGA TCC TGG CTC AGG AC - 3') and SRR182R (5'- GGT GTT CCT CMH GAT ATC TG - 3'), while those of the second PCR were SRR178F (5'- GAA CGC TGG CGG CGT GCT - 3') and SRR 179R (5' - GCG CAT TYC ACC GCT ACA CC - 3'). These primer sequences corresponded to nucleotide positions 10–29, 673–692, 30–47, and 654–673, respectively, according to the *Streptomyces ambofaciens* rDNA sequence¹⁴⁾. We employed KOD -Plus- (Toyobo Co., Ltd., Osaka, Japan) as a DNA polymerase for its high fidelity and thermostability. The first PCR reaction mixture was prepared as follows: forward and reverse primers, 0.825 pmol each; dimethyl sulfoxide, 0.55 µl; 10 × PCR buffer, 0.55 µl (KOD -Plus-); deoxynucleoside triphosphates (dNTPs), 0.55 µl (10 mM each dNTP); KOD -Plus- DNA polymerase, 0.1 µl (0.1 units); H₂O, to 5 µl. Culture broth (0.5 µl) was added to the reaction mixture as a template. PCR was performed with Gene Amp PCR System 9700 (Applied Biosystems Japan Ltd., Tokyo, Japan). To extract genome DNA from the mycelia, to denature DNA, and to activate polymerase, pre-amplification steps of 10 sec at 4 °C and 1 min at 99.9 °C were performed. Amplification was performed using 10 cycles of 15 sec of denaturing at 97 °C, 30 sec at 55 °C for primer annealing, and 2 sec at 68 °C for primer extension, followed by cooling to 4 °C. Following the first PCR reaction, the PCR reaction mixture was discarded and the second PCR performed using the same tube. The faint trace of the amplification product left in the tube then served as templates for the second PCR. The second PCR reaction mixture was prepared as follows: forward and reverse primers, 0.375 pmol each; dimethyl sulfoxide, 0.5 µl; 10 × PCR buffer, 0.5 µl; dNTPs, 0.5 µl; KOD -Plus- DNA polymerase, 0.1 µl (0.1 unit); H₂O, to 5 µl. After 1 min of denaturing at 99.9 °C, amplification was performed by 40 cycles of 15 sec of denaturing at 97 °C, 30 sec at 64 °C for primer annealing, and extension, followed by cooling to 4 °C. With no purification step, the second PCR product was used directly as a template for the sequencing reaction. Using the same tube as with second PCR, the product left in tube after discarding the reaction mixture in the preceding step was used as a template. Sequencing was performed using BigDye Terminator Cycle Sequencing Ready Reaction Kit

(Applied Biosystems Japan Ltd.) and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems Japan Ltd.). To reduce cost, the premix was used at 1/4 the strength recommended in the manufacture's instructions, reducing the reaction volume to 5 µl. The sequencing reaction mixture was prepared as follows: Terminator Ready Reaction Mix, 0.5 µl; Tris buffer (200 mM Tris HCl, 5 mM MgCl₂, pH 8.0), 1.5 µl; SRR188F, 0.8 pmol; dimethyl sulfoxide, 0.25 µl; H₂O, to 5 µl. Ethanol precipitation was performed to purify the products, using a shredded glass fiber filter GA100 (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) to absorb the supernatant.

3) Homology search and simple identification

The 16S rDNA sequence data for type strains of the order *Actinomycetales*, excluding the suborder *Micrococccineae*, was collected from DDBJ¹⁵⁾. The suborder *Micrococccineae* was excluded, since this study dealt with filamentous actinomycetes. There was relatively little sequence data available for the genus *Streptomyces*. As of October 2001, the number of approved species and subspecies in the genus *Streptomyces* was 474, whereas the number of available sequence data was 93 only (19.6%, excluding short sequences and sequences including many "n"). On the other hand, in non-streptomyces, the number of approved species and subspecies was 555 and the number of available sequence data was 468 (84.3%). To replenish the database sequence data, 16S rDNA of 74 strains (*Streptomyces*; 73, *Actinosynnema*; 1) of type strain were sequenced and registered in the database (Table 2). The in-house database was created using Excel VBA (Microsoft Corporation) based on this data and homology searches were performed (unpublished data). To confirm the results, BLAST searches were performed on the DDBJ home site.

4) Genus determination

Genus determination must be made by the polyphasic analysis depending on chemotaxonomy and morphological characteristics, in addition to phylogenetic analysis by 16S rDNA sequence. But in this study, for reasons of convenience, we employed a 16S rDNA homology value of 94% as a genus determination criterion. If an isolate demonstrated a 16S rDNA homology value with most homologous species exceeding this value, the isolate was simply identified as the genus of the species. The criterion was set at 94 % because the intragenomic homology exceeded 94 % in most genera^{16–24)}. In other cases, we confirmed the genus of an isolate from its position on the phylogenetic tree.

5) Species determination

Although the determination of bacterial species must be made by a hybridization test of genome DNA and the homology value of the 16S rDNA sequence does not become the determination criterion for species, there is close correlation between them. Keswani *et al.* showed that when the homology value of a pair of 16S rDNA sequences is 98.6%, there is a 99% probability that the

value of DNA hybridization test will be less than 70%, *i.e.*, that they are from different species²⁵⁾. For reasons of convenience, we employed a 16S rDNA homology value of 98.6% as a species determination criterion. If an isolate demonstrated a 16S rDNA homology value exceeding this value, the isolate was simple-identified as the species. Otherwise, the isolate is considered to belong to a species the sequence for which has yet to be registered in the DDBJ or which has not been described. It should be noted that the number of species which was counted based on this criterion is a minimum estimate.

Phylogenetic analysis

Phylogenetic trees were constructed by the neighbor-joining method²⁶⁾ in the Clustal X package (version 1.8)²⁷⁾.

RESULTS AND DISCUSSION

Simple identification

We were able to simple-identify 790 Malaysian isolates and 981 Japanese isolates from 1128 strains from each respective region. The success rates for simple identification (number of simple-identified strains/number of strains tried to simple-identify) of Malaysian isolates and Japanese isolates were 70.0% and 87.0%, respectively. Simple identification of these isolates was based on the result of a homology search of partial 16S rDNA sequences. The length of the sequences obtained from these isolates ranged from 402 to 589 nucleotides. Length exceeded 560 nucleotides in 85% of these sequences. In all, we attempted

simple identification of no less than 2256 isolates, succeeding with 78.5%. This rate is nearly equivalent to results obtained from simple identification by morphology^{4, 28)}. Among most isolates for which simple identification failed, the lack of success was attributable to PCR amplification failure. Improvement in PCR protocol would likely improve the success rate of simple identification. The rate for Malaysian isolates was lower than for Japanese isolates. We observed that Malaysian isolates tended to produce pigments in culture broth, pigments that may inhibit PCR amplification. This may help to explain the reasons for the lower success rate for PCR amplification of Malaysian isolates.

Comparison of the frequencies of families

About half of the species from both countries belonged to the family *Streptomyetaceae* (Malaysia; 49%, Japan; 52%). 19%, 17%, and 6% of Malaysian species belonged to *Micromonosporaceae*, *Streptosporangiaceae*, and *Nocardiaceae*, respectively. 16%, 12%, and 9% of Japanese species belonged to *Streptosporangiaceae*, *Micromonosporaceae*, and *Nocardiaceae*, respectively (Fig. 1). In all, the Malaysian and Japanese isolates represented 11 families. Seven families were found in both regions (Fig. 2). The families found only in Malaysia were *Corynebacteriaceae* (1 isolate) and *Mycobacteriaceae* (4 isolates, 2 species). The families found only in Japan were *Geodermatophilaceae* (1 isolate) and *Actinosynnemataceae* (1 isolate) (Table 3). Few isolates belonged to the families found only in one area or the other. The isolation frequen-

Table 2. Actinomycetes strains used in this study.

Name	Strain No.	Accession No.
(Type strains) <i>Streptomyces</i> 73 strains <i>Actinosynnema</i> 1 strain	IFO 3406 ^T , IFO 3197 ^T , IFO 3404, IFO 3399 ^T , IFO 13404, IFO 12247 ^T , IFO 3385 ^T , KCC A 0065 ^T , KCC S 0433 ^T , IFO 13307 ^T , IFO 13458 ^T , IFO 13103 ^T , ATCC 31133, IFO 12796 ^T , IFO 13372 ^T , IFO 13451 ^T , IFO 12825 ^T , IFO 12913 ^T , IFO 13357 ^T , IFO 13452 ^T , ATCC 25664 ^T , ATCC 27116 ^T , IFO 12899 ^T , IFO 12900 ^T , IFO 12851 ^T , IFO 13441 ^T , IFO 12860 ^T , IFO 12747 ^T , IFO 13466 ^T , IFO 12823 ^T , IFO 13425 ^T , IFO 13064 ^T , IFO 12910 ^T , IFO 12912 ^T , IFO 12767 ^T , IFO 13087 ^T , IFO 12820 ^T , IFO 12756 ^T , IFO 12901 ^T , IFO 12830 ^T , IFO 12803 ^T , IFO 12848 ^T , IFO 12891 ^T , IFO 13354 ^T , IFO 12779 ^T , IFO 12839 ^T , IFO 13977 ^T , IFO 13786 ^T , IFO 13983 ^T , IFO 13065 ^T , IFO 12905 ^T , IFO 12792 ^T , IFO 13348 ^T , JCM 4325 ^T , IFO 13484 ^T , IFO 12378 ^T , IFO 12873 ^T , IFO 12739 ^T , IFO 13448 ^T , IFO 13464 ^T , IFO 13193 ^T , ATCC 14046 ^T , IFO 13429 ^T , IFO 13045 ^T , JCM 4352 ^T , JCM 4476 ^T , JCM 4584 ^T , JCM 4861 ^T , JCM 7343 ^T , IFO 4553 ^T , JCM 4689, ATCC 29050 ^T , JCM 4185 ^T , JCM 4957 ^T	AB122707 -AB122780
Malaysian soil isolates	384A03-384H12, 387A03-387H12, 410A03-410H12, 411A03-411H12, 413A03-413H12, 415A03-415H12, 418A03-418H12, 419A03-419H12, 424A03-424H12, 428A03-428H12, 431A03-431H12, 434A03-434H12	AB122781 -AB123570
Japanese soil isolates	303A03-303H12, 306A03-306H12, 310A03-310H12, 313A03-313H12, 316A03-316H12, 319A03-319H12, 322A03-322H12, 331A03-331H12, 334A03-334H12, 337A03-337H12, 343A03-343H12, 340A03-340H12	AB123571 -AB124551

cy of strains belonging to these taxa is considered to be low in both areas when isolating from soil using the methods in this study. The coincidence of the frequencies of the families of both areas reflects the same proportions of isolates obtained by each isolation method (Table 1).

Comparison of the diversity of genera and species

With two exceptions, the Malaysian isolates belonged to 22 known genera. We will discuss the taxonomic position of the two isolates later. The Japanese isolates belonged to 22 known genera. The species counts from Malaysian isolates and Japanese isolates were 185 and 207, respectively. The ratios of species counts to simple-identified isolates were 0.234 and 0.211 with Malaysian isolates and Japanese isolates, respectively. The ratio is considered to indicate species diversity, and there appears to be no significant difference between the ratio of Malaysian actinomycetes and that of Japanese actinomycetes. Given the lack of a clear-cut result from the comparison of actinomycete diversity based on approximately 1000 isolates from each region, we can hardly conclude that the diversity of Malaysian actinomycetes exceeds Japanese actinomycetes.

Overlaps in genus and species level

In all, the Malaysian and Japanese isolates represented 30 genera. Half of these were found in both Japan and Malaysia. The remaining half occurred only in one country or the other. At the species level, 185 species were counted from Malaysia and 207 species from Japan. Combined, the two countries accounted for 344 species; but only 48 species (14%) were found in both areas (Fig. 2). Thus, the actinomycete species isolated from Japan and Malaysia show little overlap. These results are interpreted to indicate differences in species or genera frequency, not distribution.

The frequencies of the genera in each family, intra-generic phylogenetic analysis

In Japanese isolates, the predominant genus of the family *Streptosporangiaceae* was *Streptosporangium* (57%). In Malaysian isolates, the ratio of *Streptosporangium* was lower (29%), while the ratio of *Nonomuraea* increased to 45% (Fig. 3). On the phylogenetic tree, *Streptosporangium* strains form two clades²⁹⁾ (Fig. 4). Approximately about the same number of isolates from Malaysia and Japan was included in the clade, which contains *S. viridialbum*. The isolates of the other clade were almost all Japanese isolates. *Streptosporangium* was found in both countries, but it was suggested that Japanese and Malaysian isolates have partiality by the intrageneric distribution. Within the genus *Nonomuraea*, simple identification resulted in a count of up to 14 species from Malaysian isolates; these were widely distributed across the phylogenetic tree (Fig. 5). One Malaysian isolate (413B06) was simple-identified as *Nonomuraea*, due to 95% homology with *Nonomuraea*

Fig. 1. Frequencies of families.

The percentages on the graphs are the frequencies of families (above 2% are indicated).

Fig. 2. Taxonomical overlaps between Malaysian isolates and Japanese isolates.

The numbers on the graphs are the numbers of taxa.

Table 3. Actinomycetes found in Malaysia and Japan.

Family	Genus	Number of isolates		Number of species		
		Japan	Malaysia	Japan (Japan Only)	Malaysia (Malaysia Only)	Both
<i>Corynebacteriaceae</i>	<i>Corynebacterium</i>	0	1	0(0)	1(1)	0
<i>Mycobacteriaceae</i>	<i>Mycobacterium</i>	0	4	0(0)	2(2)	0
<i>Nocardiaceae</i>	<i>Nocardia</i>	24	23	17(12)	10(5)	5
	<i>Rhodococcus</i>	1	2	1(1)	2(2)	0
<i>Geodermatophilaceae</i>	<i>Blastococcus</i>	1	0	1(1)	0(0)	0
<i>Micromonosporaceae</i>	<i>Micromonospora</i>	10	17	8(8)	11(11)	0
	<i>Actinoplanes</i>	21	13	15(14)	9(8)	1
	<i>Catellatospora</i>	0	3	0(0)	2(2)	0
	<i>Couchioplanes</i>	0	1	0(0)	1(1)	0
	<i>Dactylosporangium</i>	1	15	1(1)	10(10)	0
	<i>Pilimelia</i>	0	1	0(0)	1(1)	0
	<i>Verrucosispora</i>	0	13	0(0)	2(2)	0
	<i>Virgisporangium</i>	1	0	1(1)	0(0)	0
<i>Nocardioideaceae</i>	<i>Hongia</i>	20	3	6(4)	2(0)	2
<i>Pseudonocardiaceae</i>	<i>Amycolatopsis</i>	33	5	11(10)	3(2)	1
	<i>Kibdelosporangium</i>	0	1	0(0)	1(1)	0
<i>Actinosynnemataceae</i>	<i>Saccharothrix</i>	1	0	1(1)	0(0)	0
<i>Streptomycetaceae</i>	<i>Streptomyces</i>	672	541	101(71)	90(60)	30
	<i>Kitasatospora</i>	10	1	5(5)	1(1)	0
<i>Streptosporangiaceae</i>	<i>Streptosporangium</i>	136	20	18(16)	9(7)	2
	<i>Acrocarpospora</i>	3	0	2(2)	0(0)	0
	<i>Herbidospora</i>	2	0	2(2)	0(0)	0
	<i>Microbispora</i>	1	14	1(1)	3(3)	0
	<i>Microtetrastora</i>	1	22	1(0)	2(1)	1
	<i>Nonomuraea</i>	4	69	3(0)	14(11)	3
	<i>Planotetrastora</i>	31	8	5(3)	3(1)	2
	<i>Actinomadura</i>	6	11	5(4)	5(4)	1
<i>Thermomonosporaceae</i>	<i>Cryptosporangium</i>	1	0	1(1)	0(0)	0
?	<i>Kutzneria</i>	1	0	1(1)	0(0)	0
?	undescribed genus	0	2	0(0)	1(1)	0
Total		981	790	207(159)	185(137)	48

roseola. However, on the phylogenetic tree, 413B06 is located outside the *Nonomuraea* clade. The taxonomic position of 413B06 is unclear. Recently, Nakajima *et al.* described “*Microtetrastora malaysiensis*” sp. nov. for Malaysian soil isolates³⁰. We isolated 22 *Microtetrastora* strains from Malaysia, with 14 demonstrating homology value of 99–100 % with “*Microtetrastora malaysiensis*” (Fig. 6). From Japan, only one strain (343A05) was isolated; its homology value with respect to *Microtetrastora glauca* was 99.3%.

Among *Micromonosporaceae*, the predominant genus in Japanese isolates was *Actinoplanes* (60%). This figure was lower (25%) in Malaysian isolates. *Dactylosporangium* (27%) was higher in Malaysian isolates.

In this study, most of the isolates that were simple-identified as *Actinomadura* species formed a cluster around *A. spadix* (Fig. 7). Zhang *et al.* have argued that *A. spadix* should not be included in *Actinomadura*³¹. This clustering also agreed with the cluster proposed by Wang, “Potential novel genus in *Thermomonosporaceae*”⁴.

Taxonomical position of candidate strains of novel genus

Isolates 413C04 and 413C05 offered no species with high homology values. The species with the highest homology value was *Actinomadura livida* JCM 3387^T, which exhibited a value of 92% (415/449 nucleotides). These isolates are closely related to *Thermobifida*, *Streptomonospora*, and *Nocardioopsis*, but do not form a cluster with any of them (Fig. 8). The phylogenetic tree indicates they belong to an undescribed genus that belongs or is closely related to the family *Nocardioopsaceae*. These strains were isolated from the same soil sample, same isolation method (air-drying), and same isolation plate (HV agar with erythromycin). The strains produce white aerial hyphae and spore chains and have identical morphological characteristics. Except for differences considered to be errors in sequence determination (single “n” insertion and “t” addition at the end of sequence), their sequences were identical. They may be clones. Further investigations of phylogenetic, chemotax-

Fig. 3. Frequencies of genera on families.

A: *Streptosporangiaceae*; B: *Micromonosporaceae*

The percentages on the graphs are the frequencies of genera (above 2% are indicated).

onomic, and morphological properties are required to confirm their taxonomical position.

Malaysian actinomycetes as a source for screening for novel bioactive compounds

This study found no significant difference in the numbers of actinomycete species between soil samples from Malaysia and Japan. These results are inconsistent with the differences observed between the two regions in plants and other higher organisms. But the study also found relatively little overlap between species isolated from Japan and Malaysia. Hence, a collection of actinomycetes obtained from both regions will naturally have greater taxonomic diversity than from only one. Two of the Malaysian isolates examined do not belong to any known genus, suggesting that Malaysian actinomycetes have yet to be fully investigated. Malaysian actinomycetes represent a promising source for the discovery of novel bioactive compounds.

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REFERENCES

- 1) Watve, M. G.; R. Tickoo & M. M. Jog: How many antibiotics are produced by the genus *Streptomyces*? Arch. Microbiol. 176: 386–390, 2001
- 2) Bull, A. T.; M. Goodfellow & J. H. Slater: Biodiversity as a source of innovation in biotechnology. Annu. Rev. Microbiol. 46: 219–252, 1992
- 3) Myers, N.: Threatened biotas: 'hot spots' in tropical forests. Environmentalist 8: 1–20, 1988
- 4) Wang, Y.; J. S. Zhang, J. S. Ruan, Y. M. Wang & S. M. Ali: Investigation of actinomycete diversity in the tropical rainforests of Singapore. J. Ind. Microbiol. Biotechnol. 23: 178–187, 1999
- 5) Xu, L. H.; Q. R. Li & C. Jiang: Diversity of soil actinomycetes in Yunnan, China. Appl. Environ. Microbiol. 62: 244–248, 1996
- 6) Balagurunathan, R.; L. Xu & C. Jiang: Diversity of soil actinomycetes from south India and south China. Actinomycetes 7: 89–94, 1996
- 7) Wang, Y. M.; Z. S. Zhang, X. L. Xu, J. S. Ruan & Y. Wang: *Actinopolymorpha singaporensis* gen. nov., sp. nov., a novel actinomycete from the tropical rainforest of Singapore. Int. J. Syst. Evol. Microbiol. 51: 467–473, 2001
- 8) Tsuchizaki, N.; M. Hamada & K. Hotta: Rapid characterization by colony direct PCR of distribution specificity in *Streptomyces* of *kan* gene encoding a specific aminoglycoside-3-*N*-acetyltransferase. Actinomycetol. 15: 23–29, 2001

Fig. 6. The phylogenetic tree of members belong to the genera *Microbispora*, *Microtetraspora*, *Acrocarpospora*, *Herbidospora*, and *Planotetraspora* based on partial sequence of 16S rDNA (c.a. 560 nucleotides, nucleotide position: 89–653 according to the *Streptomyces ambofaciens* rDNA sequence). The numbers on the branches are confidence limits (expressed as percentages) estimated from a bootstrap analysis with 1000 replicates (above 50% are indicated).

Fig. 7. The phylogenetic tree of members belong to the family *Thermomonosporaceae* based on partial sequence of 16S rDNA (*c.a.* 560 nucleotides, nucleotide position: 89–652 according to the *Streptomyces ambofaciens* rDNA sequence). The numbers on the branches are confidence limits (expressed as percentages) estimated from a bootstrap analysis with 1000 replicates (above 50% are indicated).

Fig. 8. Phylogenetic tree showing the position of strains 413C04 and 413C05 based on partial sequence of 16S rDNA (c.a. 560 nucleotides, nucleotide position: 89–653 according to the *Streptomyces ambofaciens* rDNA sequence). The numbers on the branches are confidence limits (expressed as percentages) estimated from a bootstrap analysis with 1000 replicates (above 50% are indicated).

- 9) Hai, L. E. & A. Z. Yahya: A note on the growth performance of plantation grown *Aquilaria malaccensis* in Peninsular Malaysia. *Journal of Tropical Forest Science* 8: 573–575, 1996
- 10) Japanese Meteorological Agency: <http://www.data.kishou.go.jp/> (Japanese).
- 11) Takeuchi, S.; M. Hayakawa & T. Yamazaki: A simplified approach to the selective isolation of motile actinomycetes. In Abstract of The 1996 Annual Meeting of The Society for Actinomycetes Japan. The Society for Actinomycetes Japan, Tokyo, Japan. (Japanese). Abstr. 2. p. 2.
- 12) Hayakawa, M.; H. Iino, S. Takeuchi & T. Yamazaki: Application of a method incorporating treatment with Chloramine-T for the selective isolation of *Streptosporangiaceae* from soil. *J. Ferment. Bioeng.* 84: 599–602, 1997
- 13) Hayakawa, M. & H. Nonomura: Humic acid-vitamin agar, a new medium for selective isolation of soil actinomycetes. *J. Ferment. Technol.* 65: 501–509, 1987
- 14) Pernodet, L. L.; F. Bocard, M. T. Alegre, J. Gagnat & M. Guérineau: Organization and nucleotide sequence analysis of a ribosomal RNA gene cluster from *Streptomyces ambofaciens*. *Gene* 79: 33–46, 1989
- 15) DNA Data Bank of Japan: <http://www.ddbj.nig.ac.jp/>
- 16) Wang, Y.; Z. Zhang & J. Ruan: Phylogenetic analysis reveals new relationships among members of the genera *Microtetraspora* and *Microbispora*. *Int. J. Syst. Bacteriol.* 46: 658–663, 1996
- 17) Koch, C.; R. M. Kroppenstedt & E. Stackebrandt: Intrageneric relationships of the actinomycete genus *Micromonospora*. *Int. J. Syst. Bacteriol.* 46: 383–387, 1996
- 18) Wang, Y.; Z. Zhang & J. Ruan: A proposal to transfer *Microbispora bispora* (Lechevalier 1965) to a new genus, *Thermobispora* gen. nov., as *Thermobispora bispora* comb. nov. *Int. J. Syst. Bacteriol.* 46: 933–938, 1996
- 19) Yassin, A. F.; F. A. Rainey, J. Burghardt, D. Gierth, J. Ungerechts, I. Lux, P. Seifert, C. Bal & K. P. Schaal: Description of *Nocardiopsis synnataformans* sp. nov., elevation of *Nocardiopsis alba* subsp. *prasina* to *Nocardiopsis prasina* comb. nov., and Designation of *Nocardiopsis antarctica* and *Nocardiopsis alborubida* as later subjective synonyms of *Nocardiopsis dassonvillei*. *Int. J. Syst. Bacteriol.* 47: 983–988, 1997
- 20) Zhang, Z.; Y. Wang & J. Ruan: A proposal to revive the genus *Kitasatospora* (Omura, Takahashi, Iwai, and Tanaka 1982). *Int. J. Syst. Bacteriol.* 47: 1048–1054, 1997
- 21) Goodfellow, M.; A. B. Brown, J. Cai, J. Chun & M. D. Collins: *Amicolatopsis japonicum* sp. nov., an actinomycete producing (S,S)-N, N'-ethylenediaminedisuccinic acid. *System. Appl. Microbiol.* 20: 78–84, 1997
- 22) Zhou, Z. H.; Z. H. Liu, Y. D. Qian, S. B. Kim & M. Goodfellow: *Saccharopolyspora spinosporotrichia* sp. nov., a novel actinomycete from soil. *Int. J. Syst. Bacteriol.* 48: 53–58, 1998
- 23) Zhang, Z.; Y. Wang & J. Ruan: Reclassification of *Thermomonospora* and *Microtetraspora*. *Int. J. Syst. Bacteriol.* 48: 411–422, 1998
- 24) Lee D. S.; E. S. Kim, Y. C. Hah: Phylogenetic analysis of the genera *Pseudonocardia* and *Actinobispora* based on 16S ribosomal DNA sequences. *FEMS Microbiol. Lett.* 182: 125–129, 2000
- 25) Keswani, J. & W. B. Whitman: Relationship of 16S rRNA

- sequence similarity to DNA hybridization in prokaryotes. *Int. J. Syst. Evol. Microbiol.* 51: 667–678, 2001
- 26) Saitou, N. & M. Nei: The neighbor joining method: a new method of constructing phylogenetic trees. *Mol. Biol. Evol.* 6: 514–525, 1987
- 27) Thompson, J. D.; T. J. Gibson, F. Plewniak, F. Jeanmougin & D. G. Higgins: The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 24: 4876–4882, 1997
- 28) Hayakawa, M.; K. Ishizawa & N. Nonomura: Distribution of rare actinomycetes in Japanese soils. *J. Ferment. Technol.* 66: 367–373, 1988.
- 29) Tamura, T.; S. Suzuki & K. Hatano: *Acrocarpospora* gen. nov., a new genus of the order *Actinomycetales*. *Int. J. Syst. Evol. Microbiol.* 50: 1163–1171, 2000
- 30) Nakajima, Y.; C. C. Ho & T. Kudo: *Microtetraspora malaysiensis* sp. nov., isolated from Malaysian primary dipterocarp forest soil. *J. Gen. Appl. Microbiol.* 49: 181–189, 2003
- 31) Zhang, Z.; T. Kudo, Y. Nakajima & Y. Wang: Clarification of relationship between the members of the family *Thermomonosporaceae* on the basis of 16S rDNA, 16S-23S rRNA internal transcribed spacer and 23S rDNA sequences and chemotaxonomic analyses. *Int. J. Syst. Evol. Microbiol.* 51: 373–383, 2001