Diversity Analysis of Actinomycetes Assemblages Isolated from Soils in Cool-temperate and Subtropical Areas of Japan

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The taxonomic diversity of actinomycetes from soils in a cool-temperate area (74 samples from Rishiri Island) and a subtropical area (94 samples from Iriomote Island) of Japan was compared. Soil samples were treated with five different pre-treatment methods and subsequently cultured on humic acid-vitamin agar, resulting in the selective isolation of 1,234 actinomycete strains. These isolates were classified using 16S rDNA sequence analysis. 16S rDNA sequence similarity values of $\geq 97\%$ and $\geq 98.7\%$ were tentatively used to identify isolates at the genus and species ranks, respectively. The actinomycete assemblages isolated comprised diverse species. Specifically, 668 of the Rishiri isolates fell into 17 families, 40 genera, and 178 species, and 566 of the Iriomote isolates belonged to 21 families, 59 genera, and 194 species. Diversity analyses based on the Shannon index revealed that there was no significant difference between the species diversity of the Rishiri isolates and that of the Iriomote isolates. However, there was little taxonomic overlap between the Rishiri isolates and the Iriomote isolates, as only 66 of the species occurred in both groups. Several species that were reportedly isolated from other tropical areas were found among the Iriomote isolates but not among the Rishiri isolates. This result suggests that climate may influence the species types of soil-inhabiting actinomycetes.

INTRODUCTION

Actinomycetes usually form a significant component of the microbial population in soil, and play a significant role in the digestion and recycling of more recalcitrant, naturally occurring organic compounds (Goodfellow & Williams, 1983). The abundance and types of these microorganisms in soil habitats vary greatly with ecological factors such as vegetation, soil pH, humus content, and water content (Williams *et al.*, 1984). Climate is also thought to be an important ecological factor influencing soil actinomycete flora, although there have been few comparisons of the actinomycete populations among areas with different climates. Xu *et al.* (1996) studied the distribution of actinomycetes in soils of Yunnan, China, and reported that their generic diversity was more complex in subtropical and tropical areas than in cool-temperate mountain and snow-mountain areas. More recently, Muramatsu *et al.* (2003) studied the taxonomic diversity of actinomycetes from forest-soils in tropical (Selangor, Malaysia) and temperate (Tokyo, Japan) areas. Using a simple identification method based on 16S rDNA partial sequence similarity, they demonstrated that actinomycetes belonging to diverse genera and species were present in both areas. Moreover, they found little taxonomic overlap between Malaysian and Japanese isolates.

The aim of this study was to investigate and compare taxonomic diversity at the genus and species level of actinomycetes from soils in cool-temperate and subtropical areas of Japan. Five different isolation methods were used to isolate actinomycetes from soils obtained from a cool-temperate area on Rishiri Island and a subtropical area on Iriomote Island. More than 1,000 actinomycetes were isolated. These were classified using a simple method based on analysis of nearly complete 16S rDNA sequences.

MATERIALS AND METHODS

Soil samples

A total of 74 samples (25 field soils, 31 forest soils, and other 18 soils from riverside, seashore, *etc.*) were collected from Rishiri, a cool-temperate island (182 km² in total area) located north-west of Hokkaido at 45.1°N and 141.1°E (Fig. 1). Annual mean temperature and annual rainfall in 2007 were 7.4°C and 859 mm, respectively (Japan Meteorological Agency: <http://www.jma.go.jp/jma/index.html>). Similarly, 95 samples (41 field soils, 12 forest soils, and other 42 soils from riverside, seashore, *etc.*) were collected at Iriomote, a subtropical island (289 km² in total area) located east of Taiwan at 24.2°N and 123.5°E (Fig. 1). Annual mean temperature and annual rainfall in 2007 were 24.2°C and 2,485 mm, respectively (JMA). Samples were

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Fig. 1. Sampling areas (Rishiri Island and Iriomote Island) in Japan.

* Annual mean temperature in 2007. ** Annual rainfall in 2007.

collected from July to September of 2007 and 2008. Each sample was passed through a 2-mm mesh sieve, air-dried at room temperature for 7 days, and subsequently used for isolation of actinomycetes (Hayakawa *et al.*, 1988).

Isolation procedures for actinomycetes

Actinomycetes were isolated from soil samples using five types of pre-treatment methods; the SDS method (Hayakawa & Nonomura, 1988), the dry-heating method (Nonomura & Ohara, 1969a; Hayakawa *et al.*, 1991a), the rehydration and centrifugation method (Hayakawa *et al.*, 2000), the chemotactic method (Hayakawa *et al.*, 1991b), and the air-drying method (Hayakawa *et al.*, 1989). Humic acid-vitamin (HV) agar (Hayakawa & Nonomura, 1987) supplemented with nalidixic acid (20 µg/ml; Sigma Chemical Co., St Louis, MO, USA) and cycloheximide (50 µg/ml; Wako Pure Chemical Ind., Osaka, Japan) was used as the isolation medium. Where appropriate, NaCl (5 or 10%) or sorbitol (10%) was added to HV agar. All plates were incubated at 30°C for 2–3 weeks. Representative actinomycete colonies growing on the isolation plates were purified by streaking onto Bennett's agar (Jones, 1949). A total of 1,234 actinomycete strains were thus obtained in pure culture.

Taxonomic analyses

Strains. All actinomycete isolates (1,234 strains) were stored on oatmeal-YGG agar (Hayakawa *et al.*, 1982) or yeast-starch agar (Nonomura & Ohara, 1969b) slants and used for taxonomic characterization.

Morphological characterization. Actinomycete isolates were grown on HA agar (Nonomura *et al.*, 1979), or on

oatmeal-YGG agar. Morphology was observed by using a light microscope or a scanning electron microscope (JSM-6500F, Jeol Ltd., Tokyo) under previously described conditions (Hayakawa *et al.*, 1996).

Phylogenetic analysis. Genomic DNA was extracted using PrepMan® ultra reagent (Applied Biosystems, CA, USA) according to the manufacturer's protocol. A nearly complete 16S rDNA sequence (1.5 Kb; positions 22–1528, according to the *Escherichia coli* numbering system; Brosius *et al.*, 1978) was amplified from each isolate by PCR following the procedure described by Tamura & Hatano (2001) and directly sequenced using an ABI Prism BigDye Terminator cycle sequencing kit (Applied Biosystems) and an ABI Model 3730 automatic DNA sequencer.

The 16S rDNA sequences determined over 1,200 bp were compared with published sequences in the TYP16 database (Chun *et al.*, 2007) using a web-based identification tool, EzTaxon, which performs similarity-based searches (<http://www.eztaxon.org>). BLAST searches of the EMBL/GenBank/DBJ database were also performed to confirm the results. Phylogenetic identity of each actinomycete isolate was then verified by constructing and evaluating phylogenetic trees. Specifically, the 16S rDNA sequences obtained from the isolates were aligned with reference sequences of a corresponding genus available from EMBL/GenBank/DBJ using the CLUSTAL_X program (Thompson *et al.*, 1997). A phylogenetic tree was constructed using neighbor-joining (NJ) tree algorithms (Saitou & Nei, 1987). The resultant NJ tree topology was evaluated by bootstrap analysis based on 1,000 replicates (Felsenstein, 1985).

Identification to genus and species level. Based on the studies of Drancourt *et al.* (2000) and Stackebrandt & Ebers (2006), we defined 16S rDNA sequence similarity values of $\geq 97\%$ and $\geq 98.7\%$ as the criteria for identification at the genus and species levels, respectively. More specifically, identification to the genus rank was defined as 16S rDNA sequence similarity of $\geq 97\%$ to that of the prototype strain sequence in the TYP16S database. Similarly, if an isolate showed $\geq 98.7\%$ 16S rDNA sequence similarity to the most homologous prototype species, the isolate was classified as the same species as that prototype species.

For those isolates that were not sufficiently similar to any reference species to be classified using the 16S rDNA-based method, taxonomic identity was inferred from the phylogenetic tree analysis. Morphological characteristics were also used to validate identity at the genus level.

Diversity analysis

The diversity of actinomycete species was examined by the Shannon diversity index (Shannon & Weaver, 1949), a general diversity index that considers both richness and evenness. The equation for the Shannon index is as follows: $H' = -\sum P_i \ln P_i$, where P_i is the number of individuals in i th species as a fraction of the total number of individuals in the sample. The Shannon diversity index was evaluated

Table 1. Isolation of actinomycetes from soils^{a)} on Rishiri Island and Iriomote Island.

Pre-treatment	Culture medium ^{b)}	No. of isolates	
		Rishiri Island	Iriomote Island
SDS and Yeast extract ^{c)}	HV agar	280	310
Dry-heating ^{d)}	HV agar	80	48
Rehydration and centrifugation ^{e)}	HV agar	206	137
Chemotaxis ^{f)}	HV agar	10	12
Air-drying	HV agar with NaCl 5%, NaCl 10%, or Sorbitol 10%	92	59
Total		668	566

^{a)}Rishiri Island: 74 samples (25 field soils, 31 forest soils, and 18 other soils from seashore, riverside, etc.). Iriomote Island: 95 samples (41 field soils, 12 forest soils, and 42 other soils from seashore, riverside, etc.).

^{b)}Contains cycloheximide (50 µg/ml) and nalidixic acid (20 µg/ml).

^{c)}Hayakawa & Nonomura (1989). ^{d)}Hayakawa *et al.* (1991a). ^{e)}Hayakawa *et al.* (2000).

^{f)}Hayakawa *et al.* (1991b).

with the Wilcoxon signed-rank test for paired samples (Flanagan, *et al.*, 2007).

RESULTS

16S rDNA-based identification

We used five different pre-treatment techniques to isolate actinomycetes from soils from Rishiri, a cool-temperate island, and Iriomote, a subtropical island. This resulted in the isolation of 1,234 actinomycete strains comprising 668 Rishiri isolates and 566 Iriomote isolates (Table 1). We sequenced more than 1,200 bp of 16S rDNA from all isolates and subsequently performed taxonomic classification to the genus and species levels. The 16S rDNA sequence similarity values of $\geq 97\%$ and $\geq 98.7\%$ were used for simplified identification of isolates at the genus and species ranks, respectively. This 16S rDNA-based method enabled the identification of 1,215 (98.5%) and 923 (74.8%) of 1,234 actinomycete isolates to genus and species levels, respectively. For those isolates that could not be identified using this method, a phylogenetic tree analysis was used to infer their taxonomic positions at the family and genus levels.

Taxonomic diversity

Actinomycete isolates fell into a variety of distantly related families, genera, and species. The Rishiri isolates (668 strains) fell into 17 families, 40 genera, and 178 species. Similarly, the Iriomote isolates (566 strains) belonged to 21 families, 59 genera, and 194 species. The species diversity was quantified using the Shannon index, and the resulting diversity values were compared with the Wilcoxon signed-rank test (Table 2). There was no significant difference ($P < 0.05$) between the Shannon diversity index of Rishiri isolates and that of Iriomote isolates.

A total of 61 genera were found among the Rishiri and Iriomote isolates (Table 3). Of these, 38 (62.2%) were found on both Rishiri and Iriomote. The most frequently

Table 2. Diversity index of actinomycete species on Rishiri (a cool-temperate island) and Iriomote (a subtropical island).

No. of species (No. of strains)		Shannon diversity index ^{a)}	
Rishiri	Iriomote	Rishiri	Iriomote
178 (507)	194 (416)	6.83 ^{b)}	7.17 ^{b)}

^{a)}Shannon & Weaver 1949.

^{b)}Values were not significantly different ($P > 0.05$; Wilcoxon signed-rank test for paired samples).

isolated genus was *Streptomyces*, followed in order by *Micromonospora*, *Actinoplanes*, *Nocardia*, *Streptosporangium*, *Dactylosporangium*, *Rhodococcus*, *Kitasatospora*, *Nonomuraea*, *Actinomadura*, *Microbispora*, *Pseudonocardia*, *Agromyces*, and *Catellatospora*. Twenty-one genera, including *Catenuloplanes*, *Krasilnikovia*, *Verrucosispora*, *Virgisporangium*, *Actinomycetospora*, *Planotetraspora*, and *Cryptosporangium*, were found only on Iriomote. Conversely, two genera, *Oerskovia* and *Actinocorallia*, were found only on Rishiri.

A total of 306 species were found among the Rishiri and Iriomote isolates. Of these, only 66 (21.6%) were found on both Rishiri and Iriomote. Species compositions of representative genera, as well as the species diversity index of each genus, are shown in Table 4. The species diversities of *Micromonospora* and *Streptomyces* among the Iriomote isolates were significantly higher ($P < 0.05$) than those of the Rishiri isolates. On the contrary, diversity of *Kitasatospora* species among Rishiri isolates was significantly higher ($P < 0.05$) than that of the Iriomote isolates. Among the Rishiri *Streptomyces* isolates, strains of *S. mauvecolor* (13 strains), *S. mirabilis* (13 strains), and *S. olivochromogenes* (25 strains), all of which belong to the *S. diastaticus* phenotypic cluster of Williams *et al.* (1983), were found frequently. Several non-*Streptomyces* species, such as *Actinoplanes philippinensis* (Couch, 1950), *Actinomadura*

Table 3. Actinomycete genera found in soils on Rishiri (a cool-temperate island) and Iriomote (a subtropical island).^{a)}

Family ^{b)}	Genus	No. of isolates		No. of species		
		Rishiri	Iriomote	Rishiri	Iriomote	Both islands
<i>Actinosynnemataceae</i>	<i>Lechevalieria</i>		1 (1)			
	<i>Lentzea</i>	2	2 (1)	2	1	
	Unidentified	1 (1)				
<i>Brevibacteriaceae</i>	<i>Brevibacterium</i>		1		1	
<i>Catenulisporaceae</i>	<i>Catenulispora</i>	1 (1) ^{c)}	1 (1) ^{c)}			
<i>Cellulomonadaceae</i>	<i>Cellulomonas</i>	3	1	3	1	
	<i>Oerskovia</i>	5		2		
	Unidentified	2 (2)	1 (1)			
<i>Cryptosporangiaceae</i>	<i>Cryptosporangium</i>		3		2	
<i>Glycomycetaceae</i>	<i>Glycomyces</i>		1		1	
<i>Intrasporangiaceae</i>	<i>Humihabitans</i>	1	4	1	1	(1) ^{d)}
	<i>Intrasporangium</i>		1		1	
	<i>Oryzihumus</i>		2		1	
	<i>Phycococcus</i>		1 (1)			
	<i>Terrabacter</i>	7	2	3	2	(1)
	Unidentified	1 (1)				
<i>Kineosporiaceae</i>	<i>Kineosporia</i>	5	1 (1)	1		
<i>Microbacteriaceae</i>	<i>Agromyces</i>	2	19 (17)	1	2	
	<i>Labeledella</i>		1 (1)			
	<i>Microbacterium</i>	5	3 (2)	4	1	(1)
	Unidentified		1 (1)			
<i>Micrococcaceae</i>	<i>Arthrobacter</i>	7 (2)	1 (1)	3		
<i>Micromonosporaceae</i>	<i>Actinoplanes</i>	44 (20)	42 (27)	8	7	(3)
	<i>Asanoa</i>	4 (1)	12 (2)	2	2	(2)
	<i>Catellatospora</i>	12 (5)	9	5	3	(3)
	<i>Catenuloplanes</i>		7		2	
	<i>Couchioplanes</i>	2	11 (2)	1	2	(1)
	<i>Dactylosporangium</i>	21 (1)	26 (1)	2	5	(2)
	<i>Hamadaea</i>		1 (1)			
	<i>Krasilnikovia</i>		3 (1)		1	
	<i>Luedemannella</i>	1 (1)	5 (3)		1	
	<i>Micromonospora</i>	22 (1)	63 (5)	12	19	(10)
	<i>Pseudosporangium</i>	1	3	1	1	(1)
	<i>Spirilliplanes</i>		1 (1)			
	<i>Verrucosipora</i>		3 (1)		1	
	<i>Virgisporangium</i>		5 (4)		1	
	Unidentified	1 (1)	2 (2)			
<i>Mycobacteriaceae</i>	<i>Mycobacterium</i>	2	7 (3)	2	4	(1)

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Family ^{b)}	Genus	No. of isolates		No. of species		
		Rishiri	Iriomote	Rishiri	Iriomote	Both islands
<i>Nocardiaceae</i>	<i>Gordonia</i>	7 (6)	2	1	2	(1)
	<i>Nocardia</i>	52 (17)	31 (1)	13	12	(2)
	<i>Rhodococcus</i>	25 (10)	20 (8)	6	7	(3)
	Unidentified		1 (1)			
<i>Nocardioidaceae</i>	<i>Kribbella</i>	12	4	6	3	(3)
	<i>Marmoricola</i>		1 (1)			
	<i>Nocardioides</i>	3 (2)	4 (2)	1	2	
	Unidentified		1 (1)			
<i>Nocardiopsaceae</i>	<i>Nocardiopsis</i>	8 (1)	4 (1)	3	3	(1)
<i>Promicromonosporaceae</i>	<i>Cellulosimicrobium</i>	1 (1)	1		1	
	<i>Isoptericola</i>	3 (1)	16 (1)	1	3	(1)
	<i>Myceligeners</i>		1		1	
	<i>Promicromonospora</i>		1		1	
<i>Pseudonocardiaceae</i>	<i>Actinomycetospora</i>		4 (4)			
	<i>Amycolatopsis</i>	6 (2)	1 (1)	1		
	<i>Pseudonocardia</i>	5 (4)	17 (5)	1	5	(1)
	<i>Saccharopolyspora</i>	2	1	1	1	
	Unidentified	3 (3)	2 (2)			
<i>Streptomycetaceae</i>	<i>Kitasatospora</i>	27	3	7	1	
	<i>Streptacidiphilus</i>	6	2 (1)	1	1	
	<i>Streptomyces</i>	275 (65)	129 (21)	66	66	(23)
	Unidentified	3 (3)				
<i>Streptosporangiaceae</i>	<i>Acrocarpospora</i>		2 (2)			
	<i>Microbispora</i>	15 (6)	10 (3)	3	1	(1)
	<i>Microtetrastora</i>	2	4	1	1	
	<i>Nonomuraea</i>	7 (2)	21 (8)	3	7	(1)
	<i>Planotetrastora</i>		3 (3)			
	<i>Sphaerisporangium</i>	1	8	1	1	
	<i>Streptosporangium</i>	40 (1)	9 (1)	4	4	(2)
<i>Thermomonosporaceae</i>	<i>Actinocorallia</i>	1		1		
	<i>Actinomadura</i>	12	15 (2)	4	7	(1)
<i>Tsukamurellaceae</i>	<i>Tsukamurella</i>		1		1	
Total		668 (161)	566 (150)	178	194	(66)

^{a)} Isolates were identified using a phylogenetic analysis based on comparison of nearly complete 16S rDNA sequences (>1,200 bp).

^{b)} Zhi *et al.* 2009.

^{c)} Strains exhibiting <98.7% 16S rDNA sequence similarity with a deposited sequence.

^{d)} Number of species found both in Rishiri and Iriomote.

bangladeshensis (Ara *et al.*, 2008), *Dactylosporangium thailandense* (Thiemann *et al.*, 1967), *Micromonospora chersina* (Tomita *et al.*, 1992), and *M. narathiwatensis* (Thawei *et al.*, 2007), all of which have been isolated from

tropical areas, were found only among the Iriomote isolates. Conversely, *Actinoplanes cyaneus* (Terekhova, *et al.*, 1977), which had been reportedly isolated from a cold area (Siberia, Russia), was found among Rishiri isolates.

Table 4. Species composition of representative genera found in soils on Rishiri (a cool-temperate island) and Iriomote (a subtropical island).

Family	Genus & Species	No. of strains ^{a)} (Diversity index)		Family	Genus & Species	No. of strains ^{a)} (Diversity index)	
		Rishiri	Iriomote			Rishiri	Iriomote
<i>Micromonosporaceae</i>	<i>Actinoplanes</i>			<i>Pseudonocardia</i>	<i>Pseudonocardia</i>		
	<i>campanulatus</i>		3		<i>carboxydivorans</i>		3
	<i>cyaneus</i>	8	1		<i>halophobica</i>		6
	<i>digitatis</i>	4	6		Other species	1	3
	<i>durahamensis</i>	1			(Shannon index)	(0.00)	(1.90)
	<i>ferrugineus</i>	3		<i>Streptomyces</i>	<i>Kitasatospora</i>		
	<i>philippinensis</i>		1		<i>gansuensis</i>	4	
	<i>regularis</i>	5			<i>griseola</i>		3
	Other species	3	4		<i>mediocidica</i>	13	
	(Shannon index)	(2.57)	(2.42)		<i>nipponensis</i>	4	
	<i>Dactylosporangium</i>				Other species	6	
	<i>aurantiacum</i>	17	8		(Shannon index)	(2.23)*	(0.00)
	<i>fulvum</i>		9		<i>Streptomyces</i>		
	<i>matsuzakiense</i>	3	4		<i>atratus</i>	9	1
	<i>roseum</i>		3		<i>bungoensis</i>	5	4
	<i>thailandense</i>		1		<i>carpaticus</i>		3
	(Shannon index)	(0.61)	(2.03)		<i>flavotricini</i>		3
	<i>Micromonospora</i>				<i>glauciniger</i>	5	2
	<i>carbonacea</i>	1	6		<i>griseorubiginosus</i>	5	
	<i>chaiyaphumensis</i>	1	12		<i>hygroscopicus</i>	1	6
	<i>chersina</i>		4		<i>lydicus</i>	8	7
	<i>chokoriensis</i>	4	3		<i>mauvecolor</i>	13	
	<i>coxensis</i>	2	8		<i>mirabilis</i>	13	
	<i>echinospora</i>		3		<i>olivochromogenes</i>	25	1
	<i>endolithica</i>		2		<i>paucisporeus</i>	4	
	<i>matsumotoense</i>		3		<i>phaeolutei-</i>	1	3
	<i>narathiwatensis</i>		2		<i>chromatogenes</i>		
	<i>siamensis</i>	4	1		<i>prunicolor</i>	4	
	Other species	9	24		<i>psammoticus</i>		4
	(Shannon index)	(3.34)	(3.81)**		<i>rishiriensis</i>	8	
<i>Nocardiaceae</i>	<i>Nocardia</i>			<i>Streptosporangia</i>	<i>sanglieri</i>	12	2
	<i>araoensis</i>		4		<i>spiroverticillatus</i>	4	
	<i>arthritidis</i>		5		<i>xanthochromo-</i>	4	2
	<i>brasiliensis</i>	3			<i>genes</i>		
	<i>elegans</i>	3	6		Other species	89	70
	<i>harenae</i>	4			(Shannon index)	(5.38)	(5.78)*
	<i>miyunensis</i>	5			<i>Nonomuraea</i>		
	<i>niigatensis</i>	2	4		<i>candida</i>	2	5
	<i>nova</i>	9			<i>dietziae</i>		2
	<i>pseudovaccinii</i>		2		<i>kuesteri</i>		2
	Other species	9	9		Other species	3	3
	(Shannon index)	(3.31)	(3.27)		(Shannon index)	(1.52)	(2.50)
	<i>Rhodococcus</i>			<i>Streptosporangium</i>	<i>album</i>	4	
	<i>aetherivorans</i>		3				

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Family	Genus & Species	No. of strains ^{a)} (Diversity index)		Family	Genus & Species	No. of strains ^{a)} (Diversity index)	
		Rishiri	Iriomote			Rishiri	Iriomote
<i>Nocardioideaceae</i>	<i>erythropolis</i>	1	4	<i>Thermomonosporaceae</i>	<i>amethystogenes</i>	6	1
	<i>jostii</i>	3			<i>claviforme</i>		4
	<i>maanshanensis</i>	6	1		<i>vulgare</i>	27	2
	<i>wratislaviensis</i>	3	1		Other species	3	1
	<i>tukisamuensis</i>	1			(Shannon index)	(1.34)	(1.75)
	Other species	3	5		<i>Actinomadura</i>		
	(Shannon index)	(2.24)	(2.52)		<i>bangladeshensis</i>		2
	<i>Kribbella</i>				<i>citrea</i>	3	
	<i>sancticalisti</i>	3			<i>maheshkhaliensis</i>	7	1
	<i>sandramycini</i>	4			<i>viridis</i>		4
<i>Nocardioideaceae</i>	Other species	5	4	<i>Thermomonosporaceae</i>	Other species	2	6
	(Shannon index)	(2.36)	(1.50)		(Shannon index)	(1.55)	(2.62)

^{a)}Strains exhibiting $\geq 98.7\%$ 16S rDNA sequence similarity to a deposited sequence.

*, **Value is significantly higher than that of control (*P < 0.05, **P < 0.01; Wilcoxon signed-rank test for paired samples).

Fig. 2. Scanning electron micrographs of isolates belonging to the families *Micromonosporaceae* and *Kineosporiaceae*. (a) *Actinoplanes* sp. RI46-Co111; (b,c) *Catenuloplanes* sp. IR34-Co109; (d) *Couchioplanes* sp. IR27-RC105; (e) *Dactylosporangium* sp. IR33-SDHV105; (f) *Micromonospora* sp. IR19-RCA110; (g) *Verrucosisporea* sp. IR10-DHHV603; (h) *Virgisporangium* sp. IR08-25; (i) *Kineosporia* sp. IR32-RC107. White bars: (a, c-g), 1 µm; (h), 2 µm; (i), 10 µm.

Fig. 3. Scanning electron micrographs of isolates belonging to the families *Nocardioidaceae*, *Actinosynnemataceae*, and *Pseudonocardiaceae*. (a) *Kribbella* sp. RI51-RC105; (b) *Nocardioides* sp. IR5-RCA101; (c) *Marmoricola* sp. IR-027S3; (d) Unidentified strain R19-10; (e) *Lentzea* sp. RI50-RCA110; (f) *Actinomycetospora* sp. IY07-53; (g) *Amycolatopsis* sp. RI50-SDHV701; (h) *Pseudonocardia* sp. IR11-SDHV701; (i) *Saccharopolyspora* sp. IR-27S2. White bars: (a–c, g–i), 1 μ m; (d), 2 μ m; (f), 5 μ m; (e), 10 μ m.

Scanning electron micrographs of representative actinomycete strains belonging to the families *Micromonosporaceae*, *Kineosporiaceae*, *Nocardioidaceae*, *Actinosynnemataceae*, *Pseudonocardiaceae*, *Streptomycetaceae*, *Thermomonosporaceae*, *Nocardiopsaceae*, *Streptosporangiaceae*, and *Cryptosporangiaceae* are shown in Fig. 2 to Fig. 4. Each micrograph shows fine morphology typical of the genus. For example, the micrograph of *Catenuloplanes* sp. IR34-Co109 (Fig. 2, b and c) shows the dichotomously branched aerial hyphae that are typical of the genus.

Nucleotide sequence accession numbers

The 16S rDNA sequences of selected isolates (42 strains representing 24 genera) that showed relatively low homology (<98.5%) to prototype species or belonged to recently recognized rare genera are registered in the EMBL/GenBank/DBJ databases under accession numbers AB546272 to AB546313.

DISCUSSION

Although climate is believed to be an important

ecological factor for microbial communities, very little is known about the effects of climate on the distribution of actinomycete species in soil. This lack of ecological information may be due to the difficulty in identifying actinomycetes at the species level. Polyphasic taxonomic approaches, including phenotypic characterization, chemotaxonomy, phylogenetic analysis based on 16S rDNA sequence, and a hybridization test of genomic DNA, are required for accurate species identification of actinomycetes. For ecological studies, however, systematic analysis of many actinomycete isolates is required. Therefore, a rapid and simple identification method is essential.

Simple molecular methods, such as the restriction fragment length polymorphism (RFLP) analysis of 16S rDNA (Conville, *et al.*, 2000; Yamamura, *et al.*, 2003) and the repetitive extragenic palindromic (REP)-PCR fingerprinting technique (Olive & Bean, 1999; Yamamura *et al.*, 2004), have been suggested for characterization and classification of environmental actinomycetes. In recent years, with the progress in direct 16S rDNA sequencing techniques and the establishment of public-domain data-

Fig. 4. Scanning electron micrographs of isolates belonging to the families *Streptomycetaceae*, *Thermomonosporaceae*, *Nocardiopsaceae*, *Streptosporangiaceae*, and *Cryptosporangiaceae*. (a) *Streptomyces* sp. IR01-03; (b) *Actinocorallia* sp. RI50-DHHV301; (c) *Actinomadura* sp. RI61-DHHV302; (d) *Nocardiopsis* sp. IR-21-N1; (e) *Microtetraspora* sp. TT071-125; (f) *Nonomuraea* sp. RI69-SDHV604; (g) *Microbispora* sp. IR12-DHHV201; (h) *Streptosporangium* sp. RI60-RC101; (i) *Cryptosporangium* sp. RI31-RCA103. White bars: (a–d, f, h), 1 µm; (e, g, i), 10 µm.

bases, 16S rDNA sequence comparison has been used successfully for simple identification at the genus or species level in ecological studies of actinomycetes (Wang *et al.*, 1999; Muramatsu *et al.*, 2003; Matsumoto, *et al.*, 2006; Iwai, *et al.*, 2009). In the present investigation, we demonstrated that 16S rDNA-based identification is an efficient method of identification, with 98.5% of 1,234 actinomycete isolates being identified to the genus level and 74.8% being identified to the species level. These results are similar to those of Muramatsu *et al.*, who identified 78.5% of 2,256 actinomycete isolates to the species level by a method based on partial 16S rDNA sequence similarity. In our study, 19 isolates (1.5%) could not be identified. These strains are considered prototype isolates of new genera or species, although further studies are needed for their accurate identification.

In this study, the Shannon diversity index results demonstrated that there was no significant difference in species diversity between Rishiri isolates and Iriomote isolates (Table 2). However, the fact that there was little taxonomic overlap between the Rishiri isolates and the

Iriomote isolates (Table 3) suggests that climate may influence the species types of soil-inhabiting actinomycetes. This hypothesis is supported by the fact that several species of tropical origin were isolated only from soils on the subtropical island, Iriomote.

The total number of actinomycete genera recovered (61 genera) is much higher than that in similar studies performed in other geographic areas (Wang *et al.*, 1999; Muramatsu *et al.*, 2003). The reason for this may be the use of different pre-treatment methods. Of these methods, the SDS method was particularly successful for isolation of actinomycetes including *Streptomyces* spp. on isolation plates while restricting the growth of impeding bacterial associates. Use of the dry-heating method, the rehydration and centrifugation method, and the chemotactic method enabled selective isolation of non-streptomycete actinomycetes. In previous studies, these organisms were recovered only incidentally using conventional dilution approaches. Progress in molecular methods has enabled the detection and characterization of actinomycetes in soil without their cultivation. However, the isolation of pure

cultures of taxonomically diverse actinomycetes is still important to understand their relative abundance in the natural environment and to find novel strains that produce useful compounds (Goodfellow & Williams, 1986). The actinomycete strains obtained in the present study could be important sources of novel bioactive compounds.

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