

NOTE

Diversity of nonribosomal peptide synthetase and polyketide synthase genes in the genus *Actinoplanes* found in Mongolia

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Mongolia has an undisturbed ecosystem with rich biodiversity, but only a few research groups have focused attention on the region for its actinomycetes diversity and their antimicrobial activities.^{1,2} The genus *Actinoplanes* is representative of rare actinomycetes and the reported source of more than 120 antibiotics.³ This study was designed to assess the individual abilities of taxonomically diverse *Actinoplanes* strains, isolated from Mongolian soil, to produce biologically active compounds. First, the antimicrobial activities of culture samples were examined, using a conventional assay that was facile and suitable for preliminary screening, to check the strains' abilities to produce antibiotics. Discrimination of distinct strains and identification of the producers of already reported compounds, called dereplication, were the next important steps for efficient screening and further assessing the isolates. In contrast to culture-dependent methods, genetic analysis is not influenced by culture conditions, and thus can address genes unexpressed under normal laboratory culture conditions. As nonribosomal peptides and polyketides represent two of the major antibiotic classes prevalent in the genus *Actinoplanes*,^{4–7} two biosynthetic gene types, nonribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) genes, were analyzed to evaluate the isolates' potential for production of these types of compounds.^{8–10} In this study, the diversity of the NRPS and PKS genes in these isolates as well as their relationships in *Actinoplanes* taxonomy were investigated.

In July 2007, 39 soil samples were collected at three sampling sites in Tov, Uvs and Dornad Provinces, Mongolia, and actinomycetes were isolated from these samples. Genomic DNA was extracted using FastBreak Cell Lysis Reagent, SV Lysis Buffer and MagneSil RED (Promega Corp., Madison, WI, USA). Polymerase chain reaction (PCR) amplification of 16S rDNA was performed as described by Tamura *et al.*¹¹ Purified PCR products were directly sequenced, using an ABI Prism BigDye Terminator Cycle Sequencing Kit and a 3730xl

Genetic Analyzer (Applied Biosystems, CA, USA). For phylogenetic analysis, the 16S rDNA sequences were aligned with genus *Actinoplanes* reference sequences using the CLUSTAL_X software and a phylogenetic tree constructed using the neighbor-joining method.¹²

Adenylation (A) domain regions in NRPS genes, ketosynthase (KS) domain regions in type-I PKS genes and KS α genes in type-II PKS genes were amplified using specific primer sets described by Ayuso-Sacido *et al.*,⁸ Schermer *et al.*¹³ and Metsa-Ketela *et al.*,¹⁴ respectively. The PCR products were cloned, sequenced and searched by BLASTX on the NCBI website and phylogenetic trees were constructed.^{10,14} The substrate-specificity-conferring residues, comprising the amino-acid (aa)-binding pocket¹⁵ and the substrate aa of A domains in the NRPS genes were predicted using the PKS/NRPS Analysis Web-site (<http://nrps.igs.umaryland.edu/nrps/>).

The 398 actinomycete strains, isolated and analyzed as described above, were preserved at the Institute of Biology, Mongolian Academy of Sciences and National Institute of Technology and Evaluation (<http://www.bio.nite.go.jp/nbdc/e/>). According to their 16S rDNA sequences, these isolates were found to comprise 250 *Streptomyces* strains and 148 rare actinomycetes including 23 *Actinoplanes* strains. With the present focus on the genus *Actinoplanes*, all *Actinoplanes* isolates were selected for further analysis (Table 1). They shared 98–100% identity with known *Actinoplanes* species according to a BLAST search of their 16S rDNA sequences and a phylogenetic analysis based on their 16S rDNA sequences revealed a taxonomically diverse collection (Figure 1a).

All 23 isolates were initially tested for antimicrobial activity using agar-plug assays.¹⁶ Thirteen strains (57%) were active against Gram-positive *Bacillus subtilis* and *Staphylococcus aureus*, two (9%) of which were also active against Gram-negative *Escherichia coli*. Also, three strains (13%) exhibited anti-yeast activities against *Saccharomyces*

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cerevisiae and five strains (22%) showed antifungal activities against *Aspergillus awamori* (Table 1). Collectively, 65% of the isolates exhibited antimicrobial activity against one or more of the test organisms. Notably, two strains (MN07-A0344 and A0367) showed inhibitory effects against all test organisms, whereas nine strains (MN07-A0329, A0351, A0360, A0362, A0363, A0364, A0365, A0369 and A0373) were specifically active against Gram-positive bacteria. In addition, two strains (MN07-A0335 and A0359) showed similar antimicrobial spectra with anti-Gram-positive and antifungal activities, whereas the activity of MN07-A0330 and A0349 were specific for fungi and yeast, respectively. The antimicrobial spectra of some phylogenetically close strains (MN07-A0362, A0363, A0364 and A0365) were identical, but it was also observed that phylogenetically distant and diverse strains (MN07-A0351, A0369, A0373, A0329 and A0360) also showed similar spectra. Meanwhile, the antimicrobial spectra of MN07-A0325, A0329 and A0344 differed considerably from each other although the three strains were phylogenetically identical.

Next, PCR assays were used to detect the presence in the isolates of NRPS genes and two kinds of PKS genes, type-I PKSs, known as modular enzymes, and type-II PKSs, mostly responsible for the synthesis of aromatic polyketides. Distinct DNA amplifications possessing the expected sizes for NRPS and type-I and -II PKS genes were observed in 17, 17 and 14 of the strains, respectively. The detected genes were further investigated by cloning and sequencing of their PCR products.

Among 68 clones sequenced from the 17 NRPS-positive strains, 39 different sequences were obtained (Supplementary Table S1). A BLASTX search showed their sequence identities, relative to already published NRPS genes, ranged from 49% (58% similarity) to 83% (88% similarity). The most BLAST hits were NRPS genes found by genome-sequencing projects on actinomycetes, such as *Streptomyces* and *Pseudonocardia*, and whose metabolites have not been identified. To determine the diversity of the 39 sequences, the deduced aa sequences were compared using a phylogenetic tree (Figure 1b). Except for two clone pairs, MN07-A0344-#7/MN07-A0329-#1 and

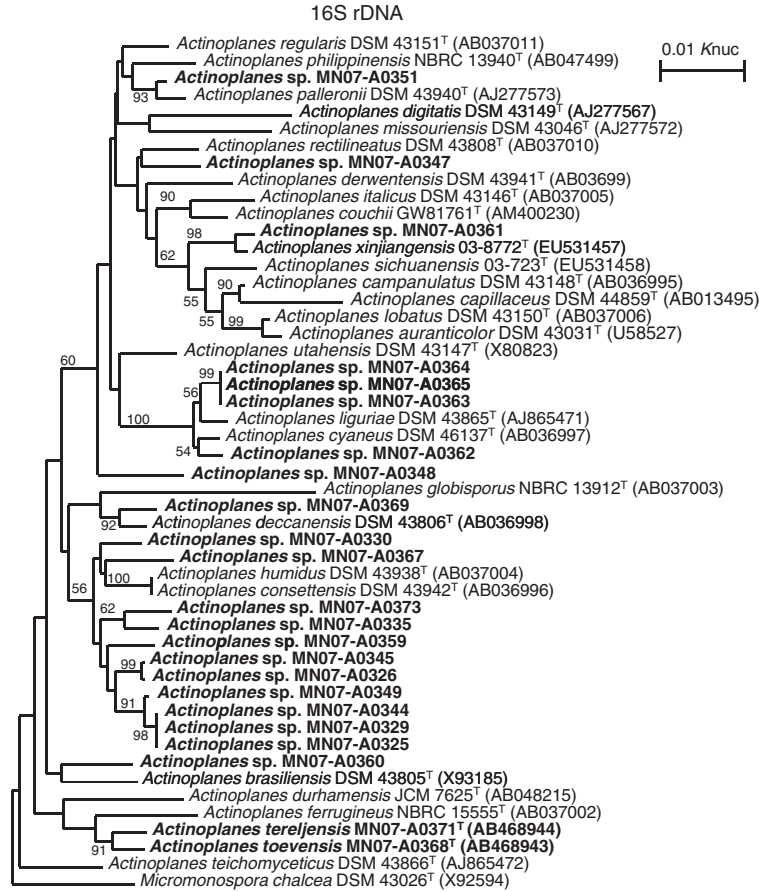
Table 1 Isolated *Actinoplanes* strains and their antimicrobial activities

Strain	Closest species based on 16S rDNA sequence (similarity)	Inhibition zone (mm) against				
		Bs	Sa	Ec	Sc	Aa
MN07-A0325	<i>A. nipponensis</i> (99%)	—	—	—	—	—
MN07-A0326	<i>A. nipponensis</i> (99%)	—	—	—	—	—
MN07-A0329	<i>A. nipponensis</i> (99%)	14	13	—	—	—
MN07-A0330	<i>A. friuliensis</i> (98%)	—	—	—	—	10
MN07-A0335	<i>A. auranticolor</i> (99%)	11	10	—	—	16
MN07-A0344	<i>A. nipponensis</i> (99%)	27	26	17	12	21
MN07-A0345	<i>A. nipponensis</i> (99%)	—	—	—	—	—
MN07-A0347	<i>A. rectilineatus</i> (98%)	—	—	—	—	—
MN07-A0348	<i>A. utahensis</i> (98%)	—	—	—	—	—
MN07-A0349	<i>A. nipponensis</i> (99%)	—	—	—	10	—
MN07-A0351	<i>A. palleronii</i> (99%)	12	12	—	—	—
MN07-A0359	<i>A. violaceus</i> (99%)	15	16	—	—	12
MN07-A0360	<i>A. digitatis</i> (98%)	10	11	—	—	—
MN07-A0361	<i>A. arizonaensis</i> (99%)	—	—	—	—	—
MN07-A0362	<i>A. cyaneus</i> (99%)	10	11	—	—	—
MN07-A0363	<i>A. garbadinensis</i> (99%)	11	11	—	—	—
MN07-A0364	<i>A. cyaneus</i> (99%)	11	10	—	—	—
MN07-A0365	<i>A. garbadinensis</i> (99%)	12	10	—	—	—
MN07-A0367	<i>A. humidus</i> (98%)	19	20	12	Tr	12
MN07-A0368	<i>A. toevensis</i> (100%)	—	—	—	—	—
MN07-A0369	<i>A. deccanensis</i> (98%)	23	20	—	—	—
MN07-A0371	<i>A. tereljensis</i> (100%)	—	—	—	—	—
MN07-A0373	<i>A. auranticolor</i> (98%)	16	13	—	—	—

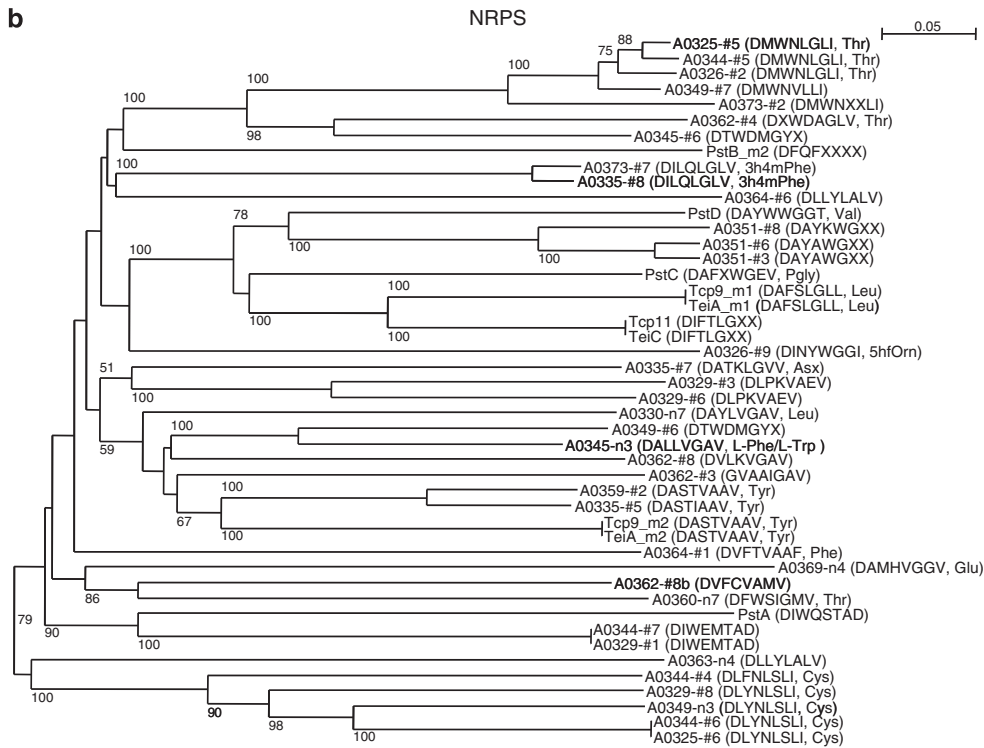
Abbreviations: Aa, *Aspergillus awamori*; Bs, *Bacillus subtilis*; Ec, *Escherichia coli*; Sa, *Staphylococcus aureus*; Sc, *Saccharomyces cerevisiae*; Tr, trace (incomplete inhibition).
—No inhibition observed.

Figure 1 Phylogenetic trees based on (a) 16S rDNA sequences, (b) A domain sequences (~230 aa) in NRPS genes, (c) KS domain sequences (~210 aa) in type-I PKS genes and (d) KS α gene sequences (~570 bp) in type-II PKS genes. Mongolian isolates bold-faced in (a); strain (MN07-) and clone no. (Supplementary Table 1) in (b, c and d); Pst and Tei/Tcp, already reported from genus *Actinoplanes*, involved in friulimicin synthesis and teicoplanin synthesis, respectively,^{6,7} added as references; specificity-conferring 8 aa residues¹⁵ and predicted substrate aa in brackets, some aa residues shown as X and some substrate aa could not be determined in the PKS/NRPS Analysis Web-site in (b); identity to closest PKS in BLASTX search in brackets in (c); reference genes with identified metabolites shown with gene name; if metabolites unidentified, strains with KS α genes in (d); bootstrap values >50% from 1000 replicates shown at nodes.

a



b



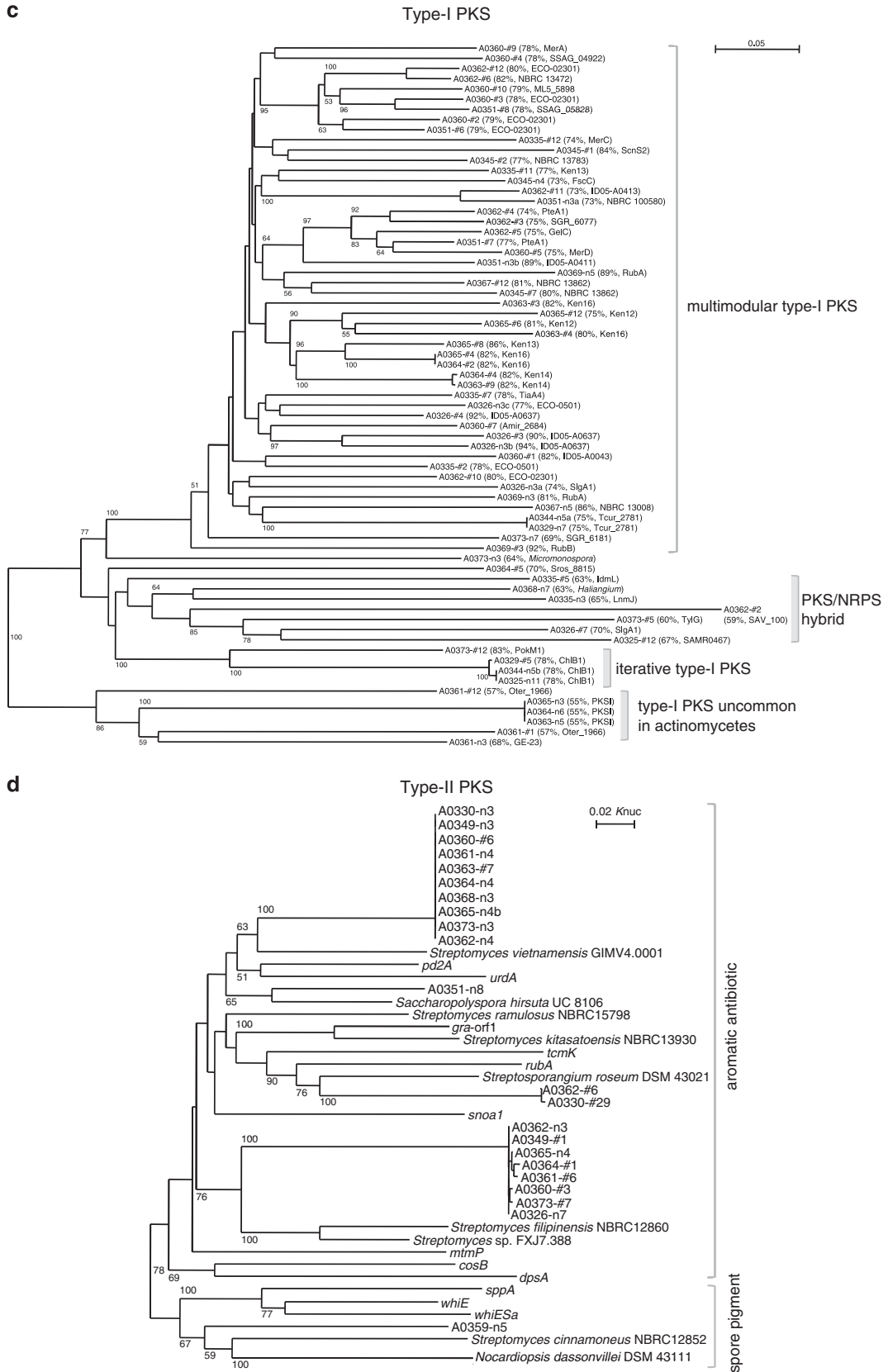


Figure 1 Continued.

MN07-A0325-#6/MN07-A0344-#6, no identical-sequence pairs were observed, suggesting NRPS gene-sequence diversity among the 17 strains. Although teicoplanins and friulimicins are known as representative nonribosomal peptide metabolites in the genus *Actinoplanes*,^{6,7} no *tcp/tei*- or *pst*-like genes corresponding to these compounds were detected in these strains. The specificity-conferring 8 aa residues of the A domains and the predicted aa substrates are also shown in Figure 1b. The substrates were closely related with the phylogenetic position of each gene sequence. Threonine (Thr)- and cysteine (Cys)-recognizing NRPS sequences were often observed, and the isolates also possessed other NRPS sequences that recognized various aa. Interestingly, phylogenetically close strains were found to often share similar NRPS sequences. For instance, similar sequences of MN07-A0355-#8 and A0373-#7 (Supplementary Table S1), which recognize 3-hydroxy-4-methoxyphenylalanine (Figures 1b) as the substrate, were observed in the phylogenetically close MN07-A0335 and A0373, respectively. Thr- and Cys-recognizing sequences were also distributed among phylogenetically close strains, such as MN07-A0325, A0344 and A0349. Indeed, the substrates of many NRPS sequences could not be predicted because their specificity-conferring 8 aa residues showed no correspondence in the related database; thus, their involvement in the synthesis of peptide compounds, including unusual aa, could be expected.

Analyses of 159 independent clones from 17 type-I PKS-positive strains yielded 70 different DNA sequences (Supplementary Table S1). Their sequence identities relative to already published type-I PKS genes ranged from 55% (64% similarity) to 94% (96% similarity). Three of six clones derived from MN07-A0329 showed over 90% identity with PKS genes of an *Actinoplanes* strain.¹⁷ MN07-A0369 possessed *rub*-like genes probably involved in rubradirin synthesis.¹⁸ Type-I PKS-coding DNA fragments, showing 74–86% identity to kendomycin synthetic genes (*ken*),¹⁹ were observed in MN07-A0335, A0363, A0364 and A0365. Except for these sequences, the novelty of many other sequences was comparatively high. Next, the sequence diversity of the isolates' type-I PKSs was checked by phylogenetic analysis (Figure 1c). Although identical or very similar sequences were infrequently observed among phylogenetically identical strains, such as MN07-A0363 and A0364, A0365 and A0325, or A0329 and A0344, most detected sequences were diverse. Phylogenetic analysis indicated that typical multimodular type-I PKS genes constituted the majority of these genes; however, unusual PKSs, likely to be PKS/NRPS hybrids or iterative types, are also often present. Furthermore, the sequences of A0365-n3/A0364-n6/A363-n5, A0361-#1 and A0361-n3 were novel and considered uncommon in actinomycetes as almost all 50 BLAST hits were type-I PKSs in non-actinomycetes (data not shown), such as *Rhodobacteraceae* bacterium and *Opitutus*. This is the first report suggesting the existence of unusual type-I PKS genes in the genus *Actinoplanes*, there being no registration of similar unusual type-I PKS genes derived from *Actinoplanes* strains in GenBank/EMBL/DDBJ.

Sequencing of 75 clones derived from 14 type-II PKS positive strains revealed 23 independent DNA sequences (Supplementary Table S1). Among them, eight strains showed amplification of multiple sequences, with MN07-A0362 yielding three kinds of sequences and MN07-A0330, A0349, A0360, A0361, A0364, A0365 and A0373 each yielding two kinds of sequences. On the other hand, only a single sequence was obtained from the remaining six strains. As type-II PKSs do not have a modular organization, a gene cluster usually encodes only a single KS α . Therefore, MN07-A0362, the seven, and the six strains grouped above were considered to possess at least three, two

and one type-II PKS gene clusters, respectively. Their sequence identities relative to already published type-II PKS genes ranged from 80% (88% similarity) to 86% (93% similarity). Most BLAST hits were KS α of *Streptomyces* sp. FXJ7.388 (accession, ADW09320) or *Streptomyces vietnamensis* (ACQ84440) and phylogenetic analysis revealed that the two types of sequences closest to them were shared by taxonomically diverse strains, such as MN07-A0361, A0364, A0373 and A0360, although four clones, A0351-n8, A0362-#6, A330-#29 and A0359-n5, were not included (Figure 1d). This suggested that their type-II PKS gene diversity was low in contrast to their NRPS and type-I PKS genes. Whereas the type-II PKS pathway is known to be involved in aromatic antibiotic synthesis, it is also involved in spore pigment synthesis; the enzymes for antibiotic biosynthesis can be phylogenetically separated from those involved in spore pigment formation.¹⁴ Except for a sequence named A0359-n5, all sequences amplified from these isolates were likely involved in antibiotic synthesis (Figure 1d).

Among the eight strains that showed no antimicrobial activity (Table 1), three strains were negative in all PCR assays. Hence, the biosynthetic potential of these three strains appeared poor. On the other hand, NRPS and/or PKS genes were amplified from five of the eight strains, suggesting that special conditions might be required to express these cryptic genes and/or that the metabolites synthesized by these pathways might not have antimicrobial activity but may possess other bioactivities. Except for these five strains, the existence of NRPS and PKS genes was well correlated with antibiotic production. As 74%, 74% and 61% of the isolates possessed NRPS, type-I and type-II PKS genes, respectively, they appear to be a useful resource with significant genetic potential.

Recent genome projects have revealed that some actinomycete families, such as *Streptomyces*, *Kitasatospora*, *Salinispora*, *Micromonospora* and *Saccharopolyspora*, regularly possess several NRPS and PKS pathways;²⁰ however, the whole genome sequence of the genus *Actinoplanes* has not been published to date (www.bio.nite.go.jp/ngac/e/project-e.html), and reports regarding NRPS and PKS gene analysis are also limited, resulting in the small number of published NRPS and PKS genes in *Actinoplanes*.^{6,7,21} To our knowledge, this is the first report focused on the genus *Actinoplanes* and the results obtained here strongly supported the conclusion that the secondary metabolite gene variety observed here in the genus *Actinoplanes* was comparable to those of the genome-sequenced genus. This is because most *Actinoplanes* strains analyzed here contained novel and diverse NRPS or PKS genes or both.

This study revealed significant genetic diversity and high antibiotic biosynthetic potential in *Actinoplanes* strains isolated from Mongolian soil samples. Structural determination of the antibiotics they produce is the important next step in further studies. Purification and structural elucidation of the metabolites was not performed here; these strains are available for researchers upon request under the Convention on Biological Diversity principle. In conclusion, the *Actinoplanes* strains investigated here appeared to have great potential as a valuable source for the future discovery of novel biologically active agents.

The accession numbers of the DNA sequences determined here are AB672891–AB673022.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (<http://www.nature.com/ja>)