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Expanding *Actinomycetota* Diversity in the TBRC Culture Collection through Metabarcoding and Simulated In Situ Cultivation of Thailand's Mekong River Microbiota

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Abstract: Culture-independent and culture-dependent approaches were employed to investigate the taxonomic diversity and biosynthetic gene cluster potential of *Actinomycetota* in the Mekong River. Through 16S rRNA gene metabarcoding, 21,103 OTUs were revealed to represent 190 genera and at least 595 species of *Actinomycetota*, including putatively novel taxa. Conventional and in situ cultivation (IC) methods provided 75 *Actinomycetota* isolates representing 72 species from 21 genera. Of these, 45 species in 4 genera were new to the Thailand Bioresource Research Center (TBRC), a collection of 20,079 *Actinomycetota* strains from 660 species. Applying both culture-independent and culture-dependent approaches to the same sample revealed greater diversity among the *Actinomycetota* in the Mekong River than one approach alone.

Keywords: *Actinomycetota*; freshwater; in situ cultivation; metabarcoding; culture collection

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1. Introduction

Actinomycetota constitutes a diverse group of Gram-positive bacteria noted for their abilities to produce antibiotics and other bioactive metabolites; almost one-fourth of such compounds discovered to date have been produced by *Actinomycetota* [1]. *Actinomycetota* also have potential uses in many other applications, including biocontrol [2–6], plant growth promotion [7–11], and bioremediation [12–14]. To foster and support such scientific research, the Thailand Bioresource Research Center (TBRC) has maintained 20,079 strains (660 species in 121 genera) of *Actinomycetota*. These comprise approximately 65% of the TBRC's bacterial collection, and 21% of the entire TBRC microbial collection. Approximately 90% of the *Actinomycetota* in the collection were acquired from several TBRC biodiversity surveys and research projects to tap the potential of *Actinomycetota* for various applications. Based on the prediction of biosynthetic gene clusters in the genome using AntiSMASH 6.0 [15], *Actinomycetota* species in the *Actinomadura*, *Actinoplanes*, *Amycolatopsis*, *Dactylosporangium*, *Kitasatospora*, *Kutzneria*, *Micromonospora*, *Nocardia*, *Nonomuraea*, *Streptomyces*, and *Streptosporangium* genera exhibit biosynthetic potential, as each of these genera contains more than 20 biosynthetic gene clusters (BGCs) with >60% homology to known clusters (Table 1). The species in these genera were predicted to contain 20–54 BGCs per genome.

The most abundant *Actinomycetota* taxa in the TBRC collection (89%) belong to the families *Micromonosporaceae*, *Nocardiaceae*, *Pseudonocardiaceae*, *Streptomycetaceae*, *Streptosporangiaceae*, and *Thermomonosporaceae*, with >80 strains for each of these families. The other

30 families comprise 1–80 strains each. Although *Actinomycetota* can be found in a variety of natural environments [16], most of those (98%) in the TBRC collection were obtained from terrestrial habitats, and 92% of these were from soils. Since *Actinomycetota* from environments other than soil also have a high potential for producing valuable compounds, it is advantageous to expand surveys and research to include other habitats. Aquatic environments have emerged as an important habitat for *Actinomycetota* with bioactivities [17–20]. Studies of bioactive compound-producing *Actinomycetota* from freshwater have lagged behind those of bioactive compound-producing *Actinomycetota* from soil ecosystems [20], most likely because of a perceived difficulty in isolating *Actinomycetota* from freshwater sources, such as because freshwater *Actinomycetota* are typically less abundant than in soils [21]. Secondary metabolites produced in freshwater habitats are thought to be highly potent in order to compensate for their dilution in water [1,19,22]. As improvements in culturing methods and advancements in DNA technology have revealed a rich diversity of *Actinomycetota* in aquatic environments [23,24], exploration of this habitat will likely yield valuable novel species and increase the number of the poorly represented taxa in the TBRC collection.

The Mekong River shows great potential as a source of novel freshwater *Actinomycetota*. As the third longest transboundary river in Asia, the river lies at the heart of the Indo-Burma biodiversity hotspot and is considered one of the richest areas of biodiversity in the world [25]. To explore freshwater *Actinomycetota* in the Mekong River, a combination of culture-independent and culture-dependent approaches was employed by a TBRC research team. An in situ cultivation (IC) method developed for the isolation and cultivation of bacteria in a simulated environment was also used to target for isolation novel strains and species of *Actinomycetota.*

Table 1. Putative BGCs of some species in *Actinomycetota* genera predicted by AntiSMASH 6.0.

Genus	Number of Species	Number of Genomes	Number of Species Containing BGCs with >60% Similarity	Number of >60% Homologous BGCs	Number of Non-Homologous BGCs	Number of BGCs Per Genome *
Acrocarpospora	4	4	4	9	53	35.25
Actinoallomurus	1	1	1	5	18	34
Actinocatenispora	1	1	1	2	4	18
Actinocorallia	2	2	2	7	28	32.5
Actinokineospora	1	1	1	1	8	32
Actinomadura	14	15	14	49	140	34.67
Actinophytocola	1	1	1	4	8	44
Actinoplanes	23	24	21	59	158	20.08
Allokutzneria	1	1	1	6	16	53
Amycolatopsis	15	16	15	68	174	35.69
Asanoa	5	5	5	13	40	17.4
Catellatospora	3	3	3	8	36	27.33
Dactylosporangium	9	10	8	33	106	29.9
Frankia	1	1	1	2	16	29
Gordonia	9	9	8	12	72	16.33
Herbidospora	3	3	3	9	23	30
Kibdelosporangium	2	2	2	12	27	50
Kineosporia	1	1	1	2	8	21
Kitasatospora	6	6	6	43	85	41.67
Kutzneria	5	6	5	34	103	54.5
Lentzea	1	1	1	8	12	40
Marinitenerispora	1	1	1	3	4	16
Microbispora	8	8	8	25	80	24.75
Micromonospora	46	48	46	136	302	20.92
Microtetraspora	3	3	3	7	28	25

Table 1. *Cont.*

Genus	Number of Species	Number of Genomes	Number of Species Containing BGCs with >60% Similarity	Number of >60% Homologous BGCs	Number of Non-Homologous BGCs	Number of BGCs Per Genome *
Mycobacterium	2	2	2	7	13	15
Nocardia	29	29	29	94	477	37.9
Nocardiopsis	1	1	1	5	6	23
Nonomuraea	14	14	14	40	139	29
Phytohabitans	5	5	5	12	35	27.8
Phytomonospora	1	1	1	2	5	17
Planobispora	4	6	4	18	63	31.33
Planomonospora	4	4	4	17	38	28.5
Planosporangium	3	3	3	5	15	15
Plantactinospora	2	2	2	3	10	19.5
Polymorphospora	1	1	1	4	11	33
Prauserella	3	4	3	12	14	20
Pseudonocardia	5	5	5	14	36	22
Pseudosporangium	1	1	1	2	4	21
Rhodococcus	5	5	5	5	30	16.2
Saccharopolyspora	4	4	4	19	23	25.5
Sinosporangium	2	3	2	14	29	34.33
Sphaerimonospora	2	2	2	6	16	18.5
Sphaerisporangium	8	8	8	22	71	27.5
Streptacidiphilus	2	2	2	9	18	24
Streptomyces	139	140	137	1401	1199	36.31
Streptosporangium	11	12	11	41	91	29.33
Thermomonospora	1	1	1	3	7	20
Tsukamurella	1	1	1	1	8	16
Virgisporangium	3	3	3	9	37	32.67
Yinghuangia	1	1	1	6	8	24

* The value in this column will represent the average number of BGCs if the number of genomes is >1.

2. Materials and Methods

2.1. Sample Collection

A total of 50 water samples, comprising 25 surface water samples (collected at 0.30 m below the surface) and 25 suspended-particle water samples (collected at 0.30 m above the riverbed), was collected along the Mekong River in Thailand at the five hydrology stations of the Department of Water Resources. These are located in Chiang Saen (CS), Chiang Khan (CK), Nong Khai (NK), Mukdahan (MD), and Khong Chiam (KC) Districts (Figure 1 and Table 2). Each hydrology station contained five sampling sites. The exact locations of the sampling sites were systematically determined using information from Acoustic Doppler Current Profiler (ADCP) machine. In addition, 15 sediment samples were collected from the riverbed, and 15 soil samples were collected on the riverbank. Furthermore, the temperature and pH values were measured at each sampling site. Water samples were stored in plastic bottles, while sediment and soil samples were placed in separate Ziploc® bags. All of the samples were kept at 4 °C during transportation from the sampling sites to the laboratory. After arriving at the laboratory, 1–10 L of each water sample was filtered prior to preparation for metabarcoding. A total of 1 L of the water sample was used for the analysis of physicochemical characteristics. The remainder of each sample was kept at 4 °C prior to processing for microbial cultivation using the IC and standard plate methods (Section 2.3).

Figure 1. Locations of the five hydrology stations of the Department of Water Resources, in Chiang Saen (CS), Chiang Khan (CK), Nong Khai (NK), Mukdahan (MD), and Khong Chiam (KC) Districts, along the Mekong River, Thailand.

Table 2. Latitude and longitude of each hydrology station location.

Hydrology Station	Latitude	Longitude
Chiang Saen	20.25691	100.101
Chiang Khan	17.90517	101.6752
Nong Khai	17.88286	102.7312
Mukdahan	16.58983	104.7398
Khong Chiam	15.33025	105.4863

2.2. DNA Extraction and 16S-Amplicon Sequencing

Between 1 and 10 L of each water sample was vacuum filtered successively through polyethersulfone (PES) membranes (0.8-, 0.4-, and 0.22-µm pore size (PALL, New York, NY, USA). DNA was extracted from cells on the membranes in the DNeasy PowerWater kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Sediment and soil samples were extracted according to the protocol described by Zhou et al. [26]. DNA concentration and purity were assessed by absorbance at 280 nm and by the 260/280 nm absorbance ratio, respectively, in a NanoDrop^{TM} spectrophotometer 2000 (Thermo Scientific, Waltham, MA, USA). DNA whose 260/280 nm absorbance ratio was between 1.8 and 2.0 was retained for library construction. Metagenomic DNA from water, sediment, and soil samples was used as the template in polymerase chain reactions (PCRs) to amplify the 16S rRNA gene taxonomic marker. The resulting amplicons were sequenced on the Illumina MiSeq platform. The amplicon sequencing library was prepared using the bacteria-specific primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) targeting the V3–V4 region of the 16S rRNA gene [27].

2.3. Isolation and Cultivation

2.3.1. Design and Development of the IC Plate

The IC plate method was designed according to Nichols et al. (2010) [28], with modifications. Briefly, an empty 200 µL-pipette tip rack (BioRobotix, Waltham, MA, USA) was polished by a polishing machine to smooth the bottom surface, and the resulting polished rack was used as the IC plate with 96 through-holes. After cleaning with water, a thin layer of silicone glue was applied to the bottom of the IC plate. The bottom of the IC plate was then covered by a 0.03 µm-pore size polycarbonate membrane (Whatman, Dassel, Germany). After letting the glue dry overnight, the IC plate was sterilized by autoclaving. Thereafter, each well was filled with 150 µL 1.5% (*w*/*v*) sterile agar. Cell suspension (1 µL) was pipetted onto the agar in each well to trap the bacterial cells. A second piece of sterile 0.03 µm-pore size polycarbonate membrane was carefully attached to the top part of the IC plate in order to cover the IC plate before the glue was left to dry overnight (Figure 2).

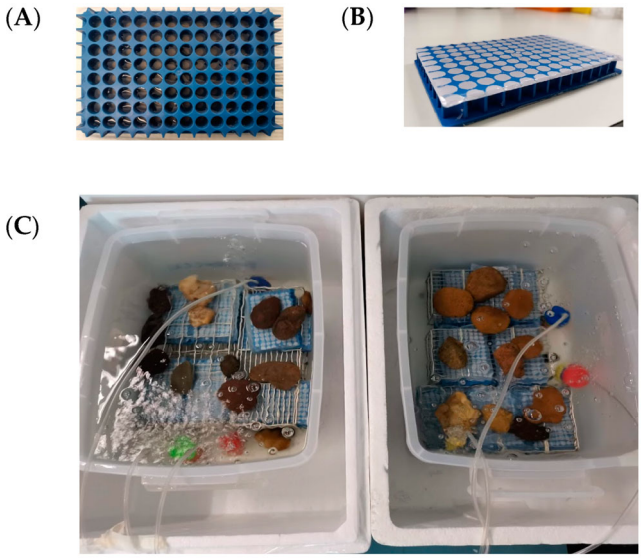

Figure 2. Design and workflow of the IC plate method. The IC plate contains 96 wells filled with 1.5% (*w*/*v*) sterile agar medium. Water samples were added onto the agar medium in each well (**A**). Then, the inoculated plate was covered by sterile 0.03 µm-pore size polycarbonate membranes (**B**), followed by incubation in a water chamber connected to an aquarium-type air pump (**C**).

2.3.2. Isolation of *Actinomycetota*

All of the water samples from the Mekong River collected at each of the hydrology stations were mixed at a 1:1 (*v*/*v*) ratio, i.e., five samples from five sampling points at each station were mixed together in equal volume. The mixed samples were serially diluted with sterile water. The IC and conventional plate methods were performed simultaneously in this study. A volume of 180 µL of diluted cell suspension (10^{-4}) was plated onto soil extract agar [29] containing 50 mg/L cycloheximide and soil extract agar containing 50 mg/L cyclohexamide, 25 mg/L nalidixic acid, and 1 mg/L terbinafine. For the IC method, a volume of 180 µL cell suspension (10^{-4}) was diluted with sterile water to a final volume of 960 µL prior to use as an IC inoculum. Then, 1 µL of the inoculum was inoculated into each of the 96 wells of the IC plates, for a total of 10 plates. Thereafter, the IC plates were incubated in a chamber (Figure 2C) containing water collected from the Mekong River, with an air pump to draw oxygen into that water. IC plates containing cell suspension were incubated at room temperature for four weeks. After that, all of the cultured microbes from the IC plates were simultaneously transferred to (a) soil extract agar [29] containing 50 mg/L cycloheximide and (b) soil extract agar containing 50/L cyclohexamide, 25 mg/L nalidixiacid, and 1 mg/L terbinafine in 96-well agar plates. Finally, all of the agar plates were incubated at 30 ± 2 °C for 2–4 weeks to allow for growth of the *Actinomycetota*.

2.3.3. Identification and Dereplication of *Actinomycetota* Isolates

Putative colonies of both mycelial and non-mycelial *Actinomycetota* were observed by the unaided eye and under a light microscope (model CX 31; Olympus, Tokyo, Japan) with a 50×X long working distance objective lens (model SLMPLN50×; Olympus). Colonies of mycelial *Actinomycetota* were expected to be slow-growing, folded, and of chalky (opaque) or leathery appearance, and contain aerial and substrate mycelia of different colors [30]. Colonies with different morphological appearances were picked and subcultured on soil extract agar and yeast extract–malt extract agar medium (ISP-2) (BD Difco™, Franklin Lakes, NJ, USA) to obtain pure colonies. Pure cultures were preserved in 20% (*v*/*v*) glycerol at −80 °C.

Taxonomic identification of the isolates was based, first, on sequencing their 16S rRNA gene: purified genomic DNA was used as a DNA template for the amplification of the 16S rRNA gene using universal primers for *Bacteria*, BSF8/20 (5′-AGAGTTTGATCCTGGCTCAG-

3′) and REVB (5′-GGTTACCTTGTTACGACTT-3′) [31]. Each PCR (50 µL) contained 1X Phusion HF buffer (Thermo Scientific, Waltham, MA, USA), 0.5 µM each of forward and reverse primers, 0.2 mM each of dNTPs, 1 U of Phusion High-Fidelity DNA polymerase (Thermo Scientific, Waltham, MA, USA), and 25 ng of DNA template. The amplification conditions were 98 °C for 30 s, then 35 cycles of 98 °C for 10 s, 57.7 °C for 30 s, and 72 °C for 45 s; and a final extension at 72 °C for 10 min. PCR products were purified and sequenced by Macrogen, Seoul, Korea. The assembled sequences were compared with others in the EzBioCloud database [32] using Blastn ver. 2.12.0+ [33] to identify the closest matching sequences of type strains.

2.4. Analysis of Microbial Community Diversity

Sequences of the V3–V4 region of the bacterial 16S rRNA genes from the water, sediment, and soil samples were subjected to quality control using FastQC [34]. Operational taxonomic units (OTUs) were constructed by sequence clustering using CD-HIT (ver. 4.8.1) [35] with a 0.97 sequence identity threshold and word length of 10. Chimeric sequences were removed using VSEARCH version 2.18.0 [36]. Taxonomy assignment of bacterial OTUs was performed on the representative OTU sequences using QIIME2's classify-sklearn v. 2020.2.0 [37] with a confidence of 0.7 and k-mer of 7 against multiple 16S rRNA gene databases, including EzBioCloud [32], GTDB release 95 [38], and SILVA v. 132 [39]. Shannon and Chao1 diversity of each sample was calculated using vegan [40] and fossil R [41] packages.

3. Results

3.1. Culture-Independent, Metabarcoding-Based Diversity, and Composition of Actinomycetota in the Mekong River

The taxonomic distribution of *Actinomycetota* diversity in the Mekong River samples based on 16S rRNA gene metabarcoding is shown in Figure 3. Sequencing of the V3–V4 region of the 16S rRNA gene resulted in 7,030,770 reads. After quality filtering, 1,337,379 reads remained for analysis, and 21,103 OTUs of *Actinomycetota* were obtained based on >97% sequence similarity to reference sequences in the SILVA, EzBioCloud, and GTDB databases.

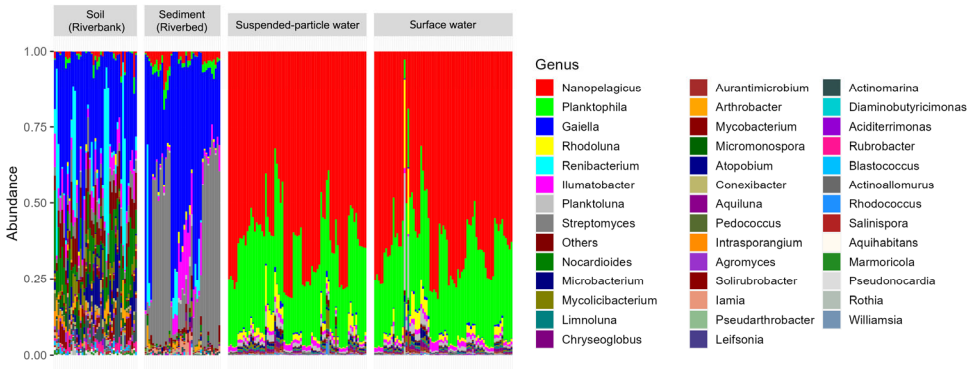

Figure 3. Relative abundance of *Actinomycetota* at the genus level in water samples (surface water and suspended-particle water), sediment, and soil samples from the Mekong River. *y*-axis indicates relative abundance level of *Actinomycetota* genera identified; *x*-axis indicates different samples.

A total of 190 *Actinomycetota* genera was identified from all of the Mekong River samples. The bacterial composition of water samples differed markedly from that of the sediment and soil samples. The dominant *Actinomycetota* in the surface water and suspended-particle water samples were *Nanopelagicus* (62%), *Planktophila* (28%), and *Rhodoluna* (2%). In the riverbed sediment samples, the dominant *Actinomycetota* genera were *Gaiella* (45%), *Streptomyces* (34%), and *Ilumatobacter* (6%), whereas the dominant genera in riverbank soil samples were *Gaiella* (31%), *Renibacterium* (11%), and *Nocardioides* (9%) (Figure 3). From these 190 *Actinomycetota* genera, 110 were not yet in the TBRC collection. Of these, 11 genera, including *Acidiferrimicrobium*, *Acidothermus*, *Actinomarina*, *Flaviluna*, *Gaiella*,

Limnoluna, *Nanopelagicus*, *Planktoluna*, *Planktophila*, *Raoultibacter*, and *Thermoleophilum*, are in the List of Prokaryotic names with Standing in Nomenclature (LPSN). However, *Actinomarina*, *Flaviluna*, *Limnoluna*, *Nanopelagicus*, *Planktoluna*, and *Planktophila* were categorized as "*Candidatus*" genera in the LPSN, indicating that cultivated type species have not yet been validly published under the International Code of Nomenclature of Prokaryotes (ICNP) [42].

To investigate variation in *Actinomycetota* compositions of the Mekong River water, sediment, and soil samples, the alpha-diversity of the OTUs was analyzed using Shannon's and Chao 1 diversity indices at the genus level (Figure 4). The two types of water samples (surface water and suspended-particle water) exhibited similar levels of genus richness and evenness (Figure 4A,B), while sediment and soil samples comprised different levels of diversity. The riverbank soil showed the highest richness and evenness levels of *Actinomycetota* compared to other samples, while the riverbed sediment samples contained the lowest level of *Actinomycetota* genus diversity.

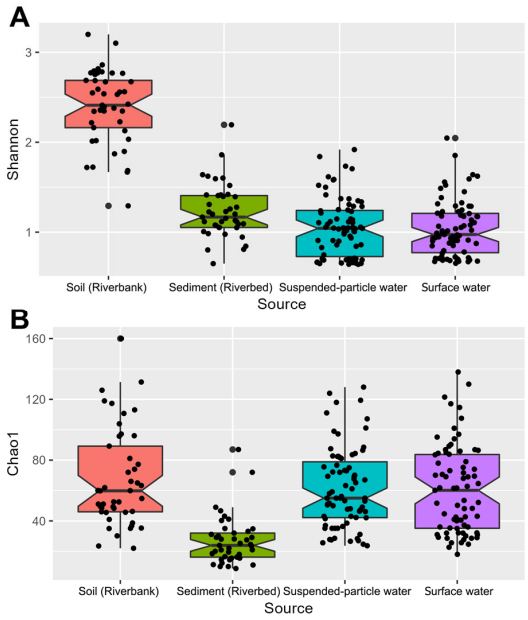

Figure 4. Boxplot of alpha-diversity of *Actinomycetota* genera identified from the Mekong samples. (**A**) Shannon and (**B**) Chao1 indices reflect the abundances in the indicated samples. The boxplot for soil (riverbank), sediment (riverbed), suspended-particle water, and surface water samples are shown in orange, green, blue, and purple, respectively. Black dots show the actual index values for each sample.

3.2. Culture-Dependent Isolation of Actinomycetota in the Mekong River

Actinomycetota in the Mekong River were also recovered by culture-dependent methods using the standard plate and IC techniques. After the initial colony isolation, 75 *Actinomycetota*-like isolates were obtained. The 16S rRNA gene in each was amplified and sequenced. The 75 isolates were confirmed to be *Actinomycetota* through EZBioCloud searches, with $\geq$98% identity. These 75 isolates were affiliated with 72 species in 21 different genera, including *Actinotalea*, *Aeromicrobium*, *Agrococcus*, *Agromyces*, *Arthrobacter*, *Asanoa*, *Brevibacterium*, *Cellulomonas*, *Geodermatophilus*, *Kribbella*, *Microbacterium*, *Microbispora*, *Micromonospora*, *Mycolicibacterium*, *Nocardia*, *Nocardioides*, *Nonomuraea*, *Pseudarthrobacter*, *Rhodococcus*, *Streptomyces*, and *Williamsia*. Among the isolated species, 45 species belonging to 18 genera were new to the TBRC collection. These species were members of the *Actinotalea*, *Aeromicrobium*, *Agrococcus*, *Agromyces*, *Brevibacterium*, *Cellulomonas*, *Geodermatophilus*, *Kribbella*, *Microbacterium*, *Micromonospora*, *Mycolicibacterium*, *Nocardia*, *Nocardioides*, *Nonomuraea*, *Pseudarthrobacter*, *Rhodococcus*, *Streptomyces*, and *Williamsia* genera (Table 3).

Table 3. Summary of 75 *Actinomycetota* isolates obtained from the Mekong River.

Genus	Number of Isolates	Order	Family	Species New to TBRC
Actinotalea	2	*Micrococcales*	*Cellulomonadaceae*	*Actinotalea fermentans*
Aeromicrobium	1	*Propionibacteriales*	*Nocardioidaceae*	*Aeromicrobium erythreum*
Agrococcus	1	*Micrococcales*	*Microbacteriaceae*	*Agrococcus terreus*
Agromyces	1	*Micrococcales*	*Microbacteriaceae*	*Agromyces indicus*
Arthrobacter	1	*Micrococcales*	*Micrococcaceae*	-
Asanoa	1	*Micromonosporales*	*Micromonosporaceae*	-
Brevibacterium	1	*Micrococcales*	*Brevibacteriaceae*	*Brevibacterium frigoritolerans*
Cellulomonas	2	*Micrococcales*	*Cellulomonadaceae*	*Cellulomonas fimi*, *Cellulomonas oligotrophica*
Geodermatophilus	1	*Geodermatophilales*	*Geodermatophilaceae*	*Geodermatophilus normandii*
Kribbella	1	*Propionibacteriales*	*Kribbellaceae*	*Kribbella speibonae*
Microbacterium	2	*Micrococcales*	*Microbacteriaceae*	*Microbacterium invictum*
Microbispora	1	*Streptosporangiales*	*Streptosporangiaceae*	-
Micromonospora	18	*Micromonosporales*	*Micromonosporaceae*	*Micromonospora rifamycinica*
Mycolicibacterium	4	*Corynebacteriales*	*Mycobacteriaceae*	*Mycolicibacterium anyangense*, *Mycolicibacterium fluoranthenivorans*, *Mycolicibacterium pallens*, *Mycolicibacterium tokaiense*
Nocardia	3	*Corynebacteriales*	*Nocardiaceae*	*Nocardia grenadensis*, *Nocardia higoensis*, *Nocardia niwae*
Nocardioides	2	*Propionibacteriales*	*Nocardioidaceae*	*Nocardioides aquiterrae*
Nonomuraea	2	*Streptosporangiales*	*Streptosporangiaceae*	*Nonomuraea helvata*, *Nonomuraea lycopersici*
Pseudarthrobacter	2	*Micrococcales*	*Micrococcaceae*	*Pseudarthrobacter niigatensis*, *Pseudarthrobacter oxydans*
Rhodococcus	2	*Corynebacteriales*	*Nocardiaceae*	*Rhodococcus cerastii*, *Rhodococcus pedocola*
Streptomyces	26	*Streptomycetales*	*Streptomycetaceae*	*Streptomyces actinomycinicus*, *Streptomyces aurantiacus*, *Streptomyces badius*, *Streptomyces brasiliensis*, *Streptomyces durhamensis*, *Streptomyces echinatus*, *Streptomyces globisporus*, *Streptomyces griseoruber*, *Streptomyces mauvecolor*, *Streptomyces naganishii*, *Streptomyces nigra*, *Streptomyces panaciradicis*, *Streptomyces pluricolorescens*, *Streptomyces prasinopilosus*, *Streptomyces reticuliscabiei*, *Streptomyces rubiginosohelvolus*, *Streptomyces sannanensis*, *Streptomyces sindenensis*, *Streptomyces turgidiscabies*,
Williamsia	1	*Corynebacteriales*	*Nocardiaceae*	*Williamsia muralis*

Remarkably, the genera *Actinotalea*, *Aeromicrobium*, *Agrococcus*, and *Williamsia* were entirely new genera to the TBRC collection.

4. Discussion

Since aquatic *Actinomycetota* have not been as extensively exploited as their terrestrial counterparts [1], *Actinomycetota* from freshwater ecosystems have now garnered increased interest as a promising source of novel bioactive compounds of pharmaceutical and biotechnological importance [43,44]. Our metabarcoding results confirmed a higher relative abundance of *Actinomycetota* (in relation to other bacteria) in water samples from the Mekong River compared to sediment and soil samples. In the Mekong River's surface and suspended-particle water samples, the *Actinomycetota* families exhibiting the highest abundance were the unicellular, free-living *Nanopelagicaceae* and *Ilumatobacteraceae*. The family *Nanopelagicaceae* has been found ubiquitously in freshwater systems [45]. Whole-genome sequences of *Nanopelagicaceae* and several other *Actinomycetota* suggested that they underwent evolutionary genome reduction events, resulting in highly streamlined genomes, possibly to help limit energy consumption (*sensu* Morris et al., 2012) [46]. *Nanopelagicaceae* are unable to synthesize various vitamins and amino acids, and require reduced sulphur, implying their dependency on these nutrients and highlighting a difficulty in the long-term cultivation of *Nanopelagicaceae* [45]. Additionally, the free-living *Ilumatobacteraceae* are part of particle-associated bacterial communities in aquatic ecosystems and play important roles in organic matter decomposition [47]. The high abundance of these families in the water samples and their relatively low abundance in the sediment and soil samples imply that they are capable of active growth and reproduction in aquatic environments, rather than being simply transients from terrestrial environments [48].

In addition to the culture-independent metabarcoding approach, our investigation using the culture-dependent approach yielded 75 *Actinomycetota* strains. Most of these isolates belonged to the *Streptomycetaceae* and *Micromonosporaceae* genera, which hosted more than 50% of the *Actinomycetota* isolates. These two families also account for approximately half of the *Actinomycetota* strains in the TBRC collection, which were mostly isolated from various terrestrial sources. We provided 45 species in 19 genera to the TBRC collection, including *Actinotalea*, *Aeromicrobium*, *Agrococcus*, and *Williamsia*, all of which were entirely new genera to the collection. *Nanopelagicus*, *Planktophila*, and *Gaiella*, as well as *Streptomyces*, were dominant genera identified from the Mekong River with the culture-independent approach. However, no representatives of these genera were cultivated here. Improvement or modification of culturing techniques may be required to isolate these *Actinomycetota*.

Our study showed that the IC methods could be applied simultaneously with the standard plate method to recover *Actinomycetota* from the Mekong River, as a total of eight species (*Actinotalea fermentans*, *Cellulomonas fimi*, *Cellulomonas oligotrophica*, *Mycolicibacterium fluoranthenivorans*, *Mycolicibacterium tokaiense*, *Nocardioides aquiterrae*, *Pseudarthrobacter niigatensis*, and *Rhodococcus cerastii*) were isolated by the IC technique but not by the standard plate technique. The success of the IC technique was most likely due to the ability to allow the *Actinomycetota* to grow in conditions that mimicked their natural habitat [28,49,50]. To obtain more diverse *Actinomycetota* species from the Mekong River, a variety of isolation media suitable for *Actinomycetota* can be developed. For example, it was found that the fastidious *Nanopelagicaceae* found in freshwater environments could be isolated and maintained in the laboratory using a catalase-supplement method [51]. Moreover, from genome analysis, *Nanopelagicaceae* was categorized as auxotrophic for heme, and it was cultured by adding heme and riboflavin to the growth medium [52]. Some rare or slow-growing *Actinomycetota* in the environment cannot be easily isolated by the agar-plating method due to competition with fast-growing microbe [4,43]. Thus, a recent array of culturing strategies with high-throughput capability has uncovered these *Actinomycetota*. For example, microfluidic streak plate methods, which allow single-cell cultivation in a droplet, cultivated individual slow-growing *Actinomycetota* [53]. Additionally, culturomics tools employing multiple culture conditions in combination with proteomics analysis, e.g., MALDI-TOF

mass spectra profiling, will be beneficial in the recovery and identification of previously uncultured or rare *Actinomycetota* [54]. Using these techniques can effectively expand the richness of *Actinomycetota* in the TBRC collection and increase opportunities to discover microorganisms capable of producing new bioactive compounds.

Several reports have investigated the diversity of *Actinomycetota* in freshwater ecosystems [17,23,53,55], but the characterization of bioactive compounds produced by isolated *Actinomycetota* lags behind that of terrestrial and marine *Actinomycetota* [1,18–20]. In addition, it has been postulated that a large fraction of *Actinomycetota* genomes contain still-unexplored gene clusters with high potential for the production of secondary metabolites [56,57]. Further characterization of the BGC potentials of freshwater *Actinomycetota* identified in this work will be valuable in uncovering strains with the capability to produce secondary metabolites. The availability of whole genome sequence data and genome mining algorithms can assist the identification and annotation of BGCs. However, the accuracy of the algorithms would still depend largely on the diversity and quality of genome data in databases. It has been observed that the numbers of BGCs vary among different genera according to genetic diversity. The less well-studied genera of *Actinomycetota* should be a primary source for unraveling important BGCs that may be related to their evolution [58]. There was also a high number of non-homologous BGCs, based on the prediction results shown in (Table 1). The predicted non-homologous BGCs should be further explored to identify bioactive metabolite production capabilities. Thus, it is advantageous to identify some cryptic or silent BGCs to facilitate the discovery of new secondary metabolites. Identifying new biosynthetic gene clusters from *Actinomycetota* will require multidisciplinary approaches to avoid rediscovering the same molecules. Genome mining with the development of synthetic biology can be used to design an expression system in heterologous hosts that enables the effective and rapid finding of secondary metabolites [55]. Together with analytical metabolomics tools, they are key to unraveling hidden pathways and discovering novel bioactive compounds [59].

Our work showed that employing both culture-independent and culture-dependent approaches simultaneously is beneficial in providing a more complete picture of *Actinomycetota* species in the Mekong River and for augmenting existing *Actinomycetota* strains in the TBRC collection. With the potential of BGC prediction, *Actinomycetota* exhibit high potential for the production of bioactive compounds. Such studies will enhance the capability of the TBRC collection in terms of microbial diversity and utilization, which will effectively serve the research and industrial communities.

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