

Diversity and novelty of actinobacteria in Arctic marine sediments

Gaiyun Zhang · Tingfeng Cao · Jianxi Ying · Yanliu Yang · Lingqi Ma

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Abstract The actinobacterial diversity of Arctic marine sediments was investigated using culture-dependent and culture-independent approaches. A total of 152 strains were isolated from seven different media; 18 isolates were selected for phylogenetic analysis on the basis of their 16S rRNA gene sequences. Results showed that the 18 isolates belonged to a potential novel genus and 10 known genera including *Actinotalea*, *Arthrobacter*, *Brachybacterium*, *Brevibacterium*, *Kocuria*, *Kytococcus*, *Microbacterium*, *Micrococcus*, *Mycobacterium*, and *Pseudonocardia*. Subsequently, 172 rDNA clones were selected by restriction fragment length polymorphism analysis from 692 positive clones within four actinobacteria-specific 16S rDNA libraries of Arctic marine sediments, and then these 172 clones were sequenced. In total, 67 phylotypes were clustered in 11 known genera of actinobacteria including *Agrococcus*, *Cellulomonas*, *Demequina*, *Iamia*, *Ilumatobacter*, *Janibacter*, *Kocuria*, *Microbacterium*, *Phycococcus*, *Propionibacterium*, and *Pseudonocardia*, along with other, unidentified actinobacterial clones. Based on

the detection of a substantial number of uncultured phylotypes showing low BLAST identities (<95 %), this study confirms that Arctic marine environments harbour highly diverse actinobacterial communities, many of which appear to be novel, uncultured species.

Keywords Actinobacteria · Arctic · Diversity · Marine sediment

Introduction

Actinobacteria are Gram-positive bacteria that are morphologically and physiologically very diverse with a high GC content in their DNA, and they have proven to be particularly useful in biotechnology applications (Manivasagan et al. 2013). They produce more than half of the known bioactive compounds derived from microbial sources (Berdy 2005; Lazzarini et al. 2000), including antibiotics (Berdy 2005), immunosuppressive agents (Mann 2001), antitumor agents (Olano et al. 2009), and enzymes (Ramesh and Mathivanan 2009). Considerable evidence indicates that actinobacteria are widely dispersed throughout marine environments, appearing in marine sediments, the water column, marine snow, and even marine vertebrates and invertebrates (Ward and Bora 2006). Ocean-bottom sediments have been reported with typical microbial abundances of 10^9 /ml (Fenical and Jensen 2006), and actinobacteria usually account for up to 9 % of the cellular community of marine

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G. Zhang (✉) · T. Cao · J. Ying · Y. Yang · L. Ma
Key Laboratory of Marine Biogenetic Resources, Third
Institute of Oceanography, State Oceanic Administration,
Xiamen 361005, People's Republic of China
e-mail: zhgyun@tio.org.cn

sediments (Bull et al. 2005). Some marine ecosystems, such as the deep-sea floor and coral reefs, are estimated to have greater biological diversity than even tropical rainforests (Haefner 2003).

Early studies of actinobacteria were able to isolate them from marine environments, yet the taxa were poorly characterized (Goodfellow and Williams 1983). Greater diversity in the actinobacteria identified from marine sources has recently been achieved by using culture-based methods, especially for indigenous marine actinobacteria. Some of the isolates have represented novel genera, including *Demequina* (Yi et al. 2007), *Gulosibacter* (Park et al. 2012), *Iamia* (Kurahashi et al. 2009), *Marinactinospira* (Tian et al. 2009a), *Marisediminicola* (Li et al. 2010), *Miniimonas* (Ue et al. 2011), *Modestobacter* (Xiao et al. 2011), *Oceanisphaera* (Srinivas et al. 2012), *Paraoerskovia* (Khan et al. 2009), *Phycicola* (Lee et al. 2008), *Salinibacterium* (Han et al. 2003), *Salinispora* (Maldonado et al. 2005), *Sciscionella* (Tian et al. 2009b), and *Serinicoccus* (Yi et al. 2004).

Due to the severe climatic conditions and logistical constraints, including large travel distances for ocean research vessels, Arctic marine environments remain poorly studied when compared to oceans in other parts of the world. Over the past decade, studies of the composition and diversity of microbial communities in the Arctic have received increasing scientific attention. Based on clone libraries and the growing body of research using cultivation-independent methods, new bacterial communities have been described from various habitats, such as permafrost soil (Hansen et al. 2007), seafloor basalt (Lysnes et al. 2004), sea ice (Brinkmeyer et al. 2003), surface snow, and meltwater (Larose et al. 2010). The major bacterial phyla that have been identified in Arctic environments include *proteobacteria*, *Actinobacteria*, *Acidobacteria*, and, to a lesser extent, *Firmicutes*, *Bacteroidetes*, *Planctomycetes*, and some unclassified taxa. Their relative abundances vary significantly depending on diverse factors such as the nature of the habitat (Jankowska et al. 2005), the extent of vegetation (Mindl et al. 2007), the level of nutrients (Kastovska et al. 2005), the depth of the sample (Ravenschlag et al. 2001), and temperature (Amato et al. 2007). Despite the fact that Arctic environments support diverse microbe communities, and that members of the phylum *Actinobacteria* are among the most successful colonizers there, comparatively little is known about Arctic

actinobacterial diversity. In fact, to our knowledge this study represents the first comprehensive characterization of the *Actinobacteria* communities in the Arctic using a culture-dependent and culture-independent method.

Materials and methods

Environmental samples

Marine sediments were collected aseptically during the 4th Chinese Arctic Scientific Expedition, in Jul–Sep 2010. The sampling sites are described in Supplemental Table 1. Sediment samples for cultivation and DNA extraction were stored at 4 and -80°C , respectively. Approximately 3 months passed between sample collections and processing.

Culture-dependent growth

Sediment samples were processed with two isolation techniques—the conventional dilution plate, and the dispersion and differential centrifugation (DDC) procedure (Hopkins et al. 1991). Treated samples were then inoculated onto seven media to isolate any constituent actinobacteria. The media were assigned numerical categories as follows: medium (1) basic agar (1 l natural seawater [NSW]); (2) Gauze's medium No.1 (1 l NSW, 1 g KNO_3 , 0.5 g K_2HPO_4 , 0.5 g $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 0.01 g $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$, 20 g starch); (3) ISP 3 medium (1 l NSW, 20 g oatmeal, 1 ml trace element solution); (4) ISP 5 medium (1 l NSW, 1 g L-Asparagine, 10 g glycerin, 1 g K_2HPO_4 , 1 ml trace element solution); (5) HV medium (1 l NSW, 0.1 g humic acid, 0.02 g CaCO_3 , 0.5 g Na_2HPO_4 , 0.5 g $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 1.7 g KCl, 0.01 g $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$, 1 ml vitamin solution); (6) Arginine-glycerin medium (1 l NSW, 6 ml Glycerin, 1 g Arginine, 0.5 g K_2HPO_4 , 0.5 g $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 20 g NaCl); and (7) S1 medium (1 l NSW, 10 g starch, 2 g tryptone, 4 g yeast). The vitamin solution contained (per litre of distilled water) 200 mg biotin, 500 mg pyridoxine HCl, 500 mg thiamine HCl, 1 g riboflavin, 1 g nicotinamide, and 100 mg p-aminobenzoic acid. The trace element solution contained (per litre of distilled water) 10 mg $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$, 40 mg ZnCl_2 , 200 mg $\text{FeCl}_3\cdot 6\text{H}_2\text{O}$, 10 mg $\text{CuCl}_2\cdot 2\text{H}_2\text{O}$, 10 mg $\text{Na}_2\text{B}_4\text{O}_3\cdot 10\text{H}_2\text{O}$, and 10 mg $(\text{NH}_4)_6\text{MO}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$.

All media were adjusted to pH 7.2–7.5 and contained 15 g l⁻¹ Difco Bacto agar, in order to produce a solid medium. In addition, all media were supplemented with a final concentration of 50 µg ml⁻¹ nystatin to inhibit fungal growth and 50 µg ml⁻¹ nalidixic acid to inhibit many fast-growing Gram-negative bacteria. The inoculated plates were incubated at 28 °C for a period of 3 months. Actinobacteria colonies were identified on the basis of morphology, and isolates were subcultured onto the original isolation medium (without antibiotics). All pure strains were maintained at 4 °C and as 20 % glycerol stocks at -80 °C for long-term maintenance.

DNA extraction and 16S rRNA gene amplification of culturable isolates

Genomic DNA was extracted according to the procedure described by Sun et al. (2010). Universal bacterial primers 27F (5'-AGAGTTTGATCCTGGC TCAG-3') and 1429R (5'-TACGGCTACCTTGT TACGACTT-3') were used to amplify 16S rRNA gene (Lane 1991). Amplification products were examined with agarose gel electrophoresis and purified using the TaKaRa Agarose Gel DNA Purification Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's specifications. Sequencing reactions were carried out with an ABI 3100 DNA sequencer at Invitrogen Biotechnology Co., Ltd (Guangzhou, China).

Culture-independent DNA extraction, PCR amplification and sequencing

Total DNA was extracted from 5 to 10 g of each sample using an UltraClean Mega DNA soil kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. DNA was subjected to a second round of purification using an Agarose Gel DNA Fragment Recovery Kit Ver.2.0 (TaKaRa, Dalian, China). Actinobacterial 16S rRNA gene fragments (approximately 640 bp) were amplified using the primers S-C-Act-0235-a-S-20 (5'-CGCGCCTATCAGCTTGTTG-3') and S-C-Act-0878-a-A-19 (5'-CCGTACTCCCCAGGCGGGG-3') (Stach et al. 2003). Purified PCR products were cloned into the pMD18-T vector and then transformed into CaCl₂-competent *Escherichia coli* DH5α. Transformed cells were plated onto Luria–Bertani agar

containing 50 µg of ampicillin ml⁻¹. Transformants were screened by PCR using the primers M13 (5'-GT TTTCCCAGTCACGAC-3') and RV (5'-CAGGAAA CAGCTATGAC-3'). The amplification products of all the positive clones were digested with the restriction enzyme *Msp* I (TaKaRa) for 4 h at 37 °C, and then electrophoresed in a 2 % agarose gel. One clone was selected for sequencing from each restriction fragment length polymorphism (RFLP) type. The clones that demonstrated distinct RFLP patterns were sequenced at Guang Zhou Invitrogen Biotechnology Co., Ltd. using the primer RV.

Phylogenetic analysis and diversity estimates

The sequence data were proofread using Chromas, version 1.62 (Technelysium) and examined for the formation of chimeras using DECIPHER's Find Chimeras web tool (Wright et al. 2012). Unique clones (one from each RFLP type) were compared to 16S rRNA gene in the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm (<http://www.ncbi.nlm.nih.gov/>). They were classified at 80 % confidence levels using the RDP Classifier of the Ribosomal Database Project (RDP) Release 10 (<http://rdp.cme.msu.edu/>) (Wang et al. 2007). Nonredundant databases were used to obtain the closest matches and these were aligned using the Clustal W program to the sequences generated in this study (Aiyar 1999). Phylogenetic analyses were conducted with MEGA 5.20 (Molecular Evolutionary Genetics Analysis, Version 5.20) (Tamura et al. 2011). Tree topologies were evaluated using bootstrap analyses based on 1,000 replicates, and phylogenetic trees were generated using the neighbor-joining method. Only bootstrap values greater than 50 % were shown on the phylogenetic trees.

The clone library coverage was calculated with the equation $C = 1 - n/N$, where n is the number of unique RFLP types and N is the total number of analyzed clones (Jiang et al. 2006). The Shannon biodiversity index and Chao1 species richness index, were calculated using the program SPADE (Chao and Shen 2005), at 95 % CIs (<http://chao.stat.nthu.edu.tw/>). The phylogenetic diversity of all four libraries was compared using the program Libshuff (<http://www.mothur.org/wiki/Libshuff>), which uses the Cramer-von Mises test to identify significant differences ($p < 0.025$) among libraries.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences of representative isolates and clones were separately deposited in the GenBank database with the following accession nos: KF840227–840244 and KF840245–840311.

Results and discussion

Culturable actinobacteria diversity

Several different treatment approaches have been used in attempts to isolate actinobacteria from their various marine habitats (Hames-Kocabas and Uzel 2012; Goodfellow and Fiedler 2010). In this study, the serial dilution method and the DDC procedure were used to achieve the selective isolation of actinobacteria from 21 Arctic sediment samples (Supplemental Table 1), producing a total of 152 strains with different morphologies. Even though sediment-processing methods were not applied identically to all samples, the DDC procedure (resulting in 126 isolates) proved to be a highly successful way to cultivate actinobacteria, compared with the reciprocal shaking technique (26 isolates). These results are in line with those of previous studies, which showed that the DDC procedure was more effective than classical shaking techniques for extracting actinomycete propagules from soil samples (Gontang et al. 2007; Atalan et al. 2000; Sembiring et al. 2000). In addition, significant differences were observed in the total number of isolates recovered among the seven different media. S1 produced the highest recovery with 64 isolates, followed by ISP 5 (20 isolates), Arginine-glycerin medium (20 isolates), basic agar (18 isolates), Gauze's medium No.1 (13 isolates), ISP 3 (12 isolates), and HV (5 isolates). The results indicated that media composed of relatively simple nutrients yielded more cultured actinobacteria, which is consistent with the results from previous studies (Qin et al. 2012; Gontang et al. 2007).

Among all the colonies, 152 putative actinobacterial strains were selected from the isolation plates and sub-cultured for further analysis. The 16S rRNA gene of the strains were sequenced, and they were assigned to genera after BLAST analysis. On the basis of either partial or complete 16S rRNA gene sequences, a total of 18 different taxa were identified definitively. The

nearly complete 16S rRNA gene sequences of representative isolates from each of these taxa were then used to construct phylogenetic trees (Fig. 1). The isolated strains displayed considerable diversity, which was distributed among three suborders: *Pseudonocardineae*, *Corynebacterineae*, and *Micrococci-neae* within the class *Actinobacteria*, including 10 families and 11 genera, more than 18 species (Table 1). Of the isolated actinobacteria, 48 % (73/152) belonged to the genus *Pseudonocardia*. Surprisingly, all isolates showed 99–100 % sequence identity to *Pseudonocardia carboxydivorans* Y8^T. The strain Y8^T, capable of oxidizing carbon monoxide, was isolated from a soil sample collected from the roadside in Seoul, Korea (Park et al. 2008). Lin et al. (2011) reported that a *P. carboxydivorans* isolate from a petroleum-contaminated soil can decompose quinoline. The second dominant group within the culturable actinobacteria was *Mycobacterium*. This genus represented 20 % (31/152) of the specimens; 20 isolates showed 99–100 % sequence identity to *Mycobacterium poriferae* ATCC 35087^T, a scotochromogenic, rapidly growing species isolated from a marine sponge (Padgitt and Moshier 1987). Six isolates showed high sequence identity (99.5–99.8 %) to *M. porcinum* CIP 105392^T, a porcine pathogen (Tsukamura et al. 1983). Three isolates showed 99.8 % homology to *M. neoaurum* ATCC 25795^T and the other two showed 99 % homology to *M. parafortuitum* DSM 43528^T. *Mycobacteria* are used widely in bioremediation of aged contaminated sites, and are reported to have good catabolic efficiency on PAHs of up to five benzene rings (Haritash and Kaushik 2009). For example, *Mycobacterium* sp. strain KR2 was able to utilize pyrene as sole source of carbon and energy, metabolizing up to 60 % of the pyrene added (0.5 mg ml⁻¹) within 8 days at 20 °C (Rehmann et al. 1998). *Mycobacterium* sp. strain SWU-4, isolated from a petroleum-contaminated site in Thailand, was able to degrade a wide variety of organosulfur compounds including thiophene, bromo (alpha) thiophene, and 3-methylthiophene in liquid minimum medium at 50 °C (Watanapokasin et al. 2002). The majority of actinobacteria that we isolated from Arctic marine sediments were *Pseudonocardia* and *Mycobacterium* species. These taxa may be associated with environmental pollution of the Arctic (Riget et al. 2010). The third dominant group was *Brevibacterium*, with 13 % of isolates (20/152) belonging to this genus. The

Fig. 1 Neighbour-joining phylogenetic tree of the selected representative actinobacteria isolates from the Arctic showing bootstrap values (1,000 replications). The nearest type strains revealed through a BLAST search of non-redundant nucleotide sequences in the public databases are presented in the tree. Nucleotide sequence accession nos are given in brackets. Bootstrap values of >50 % (for 1,000 iterations) are shown. The scale bar corresponds to 0.01 substitutions per nucleotide position

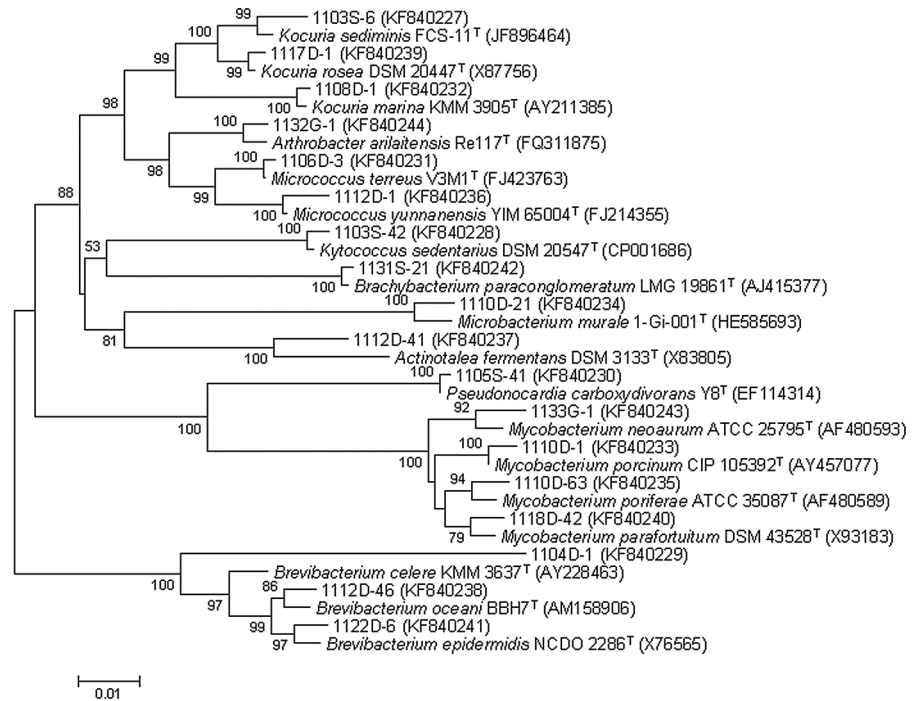


Table 1 Sequence analysis of representative actinobacteria strains isolated from Arctic marine sediments based on 16S rRNA sequencing

Representative strain	Sequence length (bp)	Number of strains	Nearest type strain	Sequence identity (%)
1103S-42 (KF840228)	1,483	2	<i>Kytococcus sedentarius</i> DSM 20547 ^T	99.58
1103S-6 (KF840227)	1,538	5	' <i>Kocuria sediminis</i> ' FCS-11 ^T	98.81
1104D-1 (KF840229)	1,412	1	<i>Brevibacterium celere</i> KMM 3637 ^T	92.24
1105S-41 (KF840230)	1,485	73	<i>Pseudonocardia carboxydivorans</i> Y8 ^T	99.86
1106D-3 (KF840231)	1,490	3	<i>Micrococcus terreus</i> V3M1 ^T	99.72
1108D-1 (KF840232)	1,487	1	<i>Kocuria marina</i> KMM 3905 ^T	99.58
1110D-1 (KF840233)	1,494	6	<i>Mycobacterium porcinum</i> CIP 105392 ^T	99.59
1110D-21 (KF840234)	1,488	2	<i>Microbacterium murale</i> 1-Gi-001 ^T	98.75
1110D-63 (KF840235)	1,489	20	<i>Mycobacterium poriferae</i> ATCC 35087 ^T	98.99
1112D-1 (KF840236)	1,423	4	<i>Micrococcus yunnanensis</i> YIM 65004 ^T	99.09
1112D-41 (KF840237)	1,486	2	<i>Actinotalea fermentans</i> DSM 3133 ^T	96.96
1112D-46 (KF840238)	1,422	11	<i>Brevibacterium oceani</i> BBH7 ^T	98.91
1117D-1 (KF840239)	1,484	1	<i>Kocuria rosea</i> DSM 20447 ^T	99.66
1118D-42 (KF840240)	1,082	2	<i>Mycobacterium parafortuitum</i> DSM 43528 ^T	99.06
1122D-6 (KF840241)	1,418	9	<i>Brevibacterium epidermidis</i> NCDO 2286 ^T	98.97
1131S-21 (KF840242)	1,479	5	<i>Brachybacterium paraconglomeratum</i> LMG 19861 ^T	99.71
1132G-1 (KF840244)	1,422	2	<i>Arthrobacter arilaitensis</i> Re117 ^T	99.26
1133G-1 (KF840243)	1,482	3	<i>Mycobacterium neoaurum</i> ATCC 25795 ^T	98.85

isolate 1104D-1 showed the greatest 16S rRNA sequence similarity (92 %) with that of a known *Brevibacterium* species, and it formed a distinct branch within the phylogenetic cluster composed of the members of the genus *Brevibacterium*; thus, likely represents a new genus. The remaining 18 % (27/152) belonged to seven other actinobacterial genera: *Kocuria* (7 strains), *Micrococcus* (7 strains), *Brachybacterium* (5 strains), *Microbacterium* (2 strains), *Actinotalea* (2 strains), *Kytococcus* (2 strains), and *Arthrobacter* (2 strains).

Using the spread plate technique, members of the eight actinobacterial genera, viz. *Streptomyces*, *Arthrobacter*, *Rhodococcus*, *Saccharothrix*, *Rathayibacter*, *Micrococcus*, *Nocardia*, and *Kribbella*, were isolated from rhizospheric soil of seven plant species collected from the Arctic Yellow River Station (Teng et al. 2009). In addition, eight major groups of actinobacteria (*Arthrobacter*, *Micrococcaceae*, *Rhodococcus*, *Streptomyces*, *Microbacterium*, *Cryobacterium*, *Brevibacterium*, and *Nocardioides*) were also isolated from Arctic sediments by Kochkina et al. (2001). In the present work, 11 groups of actinobacteria were isolated. Though actinobacteria species and abundances varied significantly in the samples obtained by different researchers, the overall results reflect the taxonomic diversity and culturability of actinobacteria from the Arctic.

Culture-independent actinobacterial diversity

Studies in many environments have used culture-independent techniques to assess actinobacteria communities based on 16S rRNA gene analysis, using universal actinobacteria primers (Holmfeldt et al. 2009; Sun et al. 2010). To better understand the diversity of actinobacteria in the Arctic region, four 16S rRNA gene clone libraries were constructed from the CO5, S23, BN09, and BN13 samples. A total of 727 clones (186, 180, 183, and 178 for CO5, BN13, BN09, and S23, respectively) were sampled randomly, and amplified with PCR using sequencing primers RV and M13. Of the 692 positive clones analyzed, 180, 168, 179, and 165 clones were obtained from CO5, BN13, BN09, and S23, respectively, and the amplification products were characterized preliminarily with RFLP analysis. A total of 172 unique RFLP types (35, 46, 43, and 48 from CO5, BN13, BN09, and S23, respectively) were identified. One clone was selected for sequencing from

each RFLP type. After eliminating 47 chimeric sequences, the remaining 125 partial 16S rRNA gene sequences were submitted to the RDP for preliminary taxonomic assignments. As has been reported elsewhere (Smith et al. 2006; Babalola et al. 2009), the majority of the phylotypes identified in the culture-independent study fell into uncultured classes. Of the 125 clones, 28 could not be identified with 80 % confidence using the classifier tool. This suggested that many clones may represent novel taxa. A further 30 clones were most closely related to the non-actinobacterial phyla *Proteobacteria* (25 clones), *Planctomycetes* (1), *Verrucomicrobia* (1), *Nitrospira* (1), *Acidobacteria* (1), and the candidate phylum WS3 (1). Interestingly, these non-actinobacterial phyla identified in this study with the actinobacteria-specific primers, have been reported in many papers by other researchers at the time of the analysis of the culture-independent actinobacterial community with the same primers (Babalola et al. 2009; Sun et al. 2010). The results showed that this primer set (S-C-Act-235-a-S-20 and S-C-Act-878-a-A-19) may produce non-specific amplification, or may not be specific for all *Actinobacteria*. As the primers were originally designed to be diagnostic for *Actinobacteria* 16S rRNA gene based upon the known actinobacterial strains available in the GenBank database (Stach et al. 2003), more specific primers should be designed as more and more actinobacteria are isolated, to achieve greater efficacy. In addition, a nested PCR protocol could be used with an external primer that does not amplify non-target 16S rDNA, resulted in 99 % of the tested clones being of actinobacteria origin (Stach et al. 2003).

The remaining 67 clones belonged to the class *Actinobacteria*, of which 25 clones were affiliated with unclassified actinobacterial groups. The other 42 clones could be grouped into specific taxa, viz. the genera *Ilumatobacter* (14 clones), *Iamia* (5), *Cellulomonas* (1), *Demequina* (3), *Janibacter* (3), *Phycococcus* (1), *Agrococcus* (1), *Microbacterium* (4), *Kocuria* (2), *Propionibacterium* (2), and *Pseudonocardia* (6). The phylogenetic trees were constructed between 67 clones and their nearest neighbours (Fig. 2). Among the 67 different representative clones (Supplemental Table 2), 40 clones (59.7 %) were less than 97 % identical to the closest type strains in the database, and 32 clones (47.8 %) exhibited less than 95 % similarity to other sequences. Thus, the primary data indicated that highly diverse actinobacterial populations were present in the

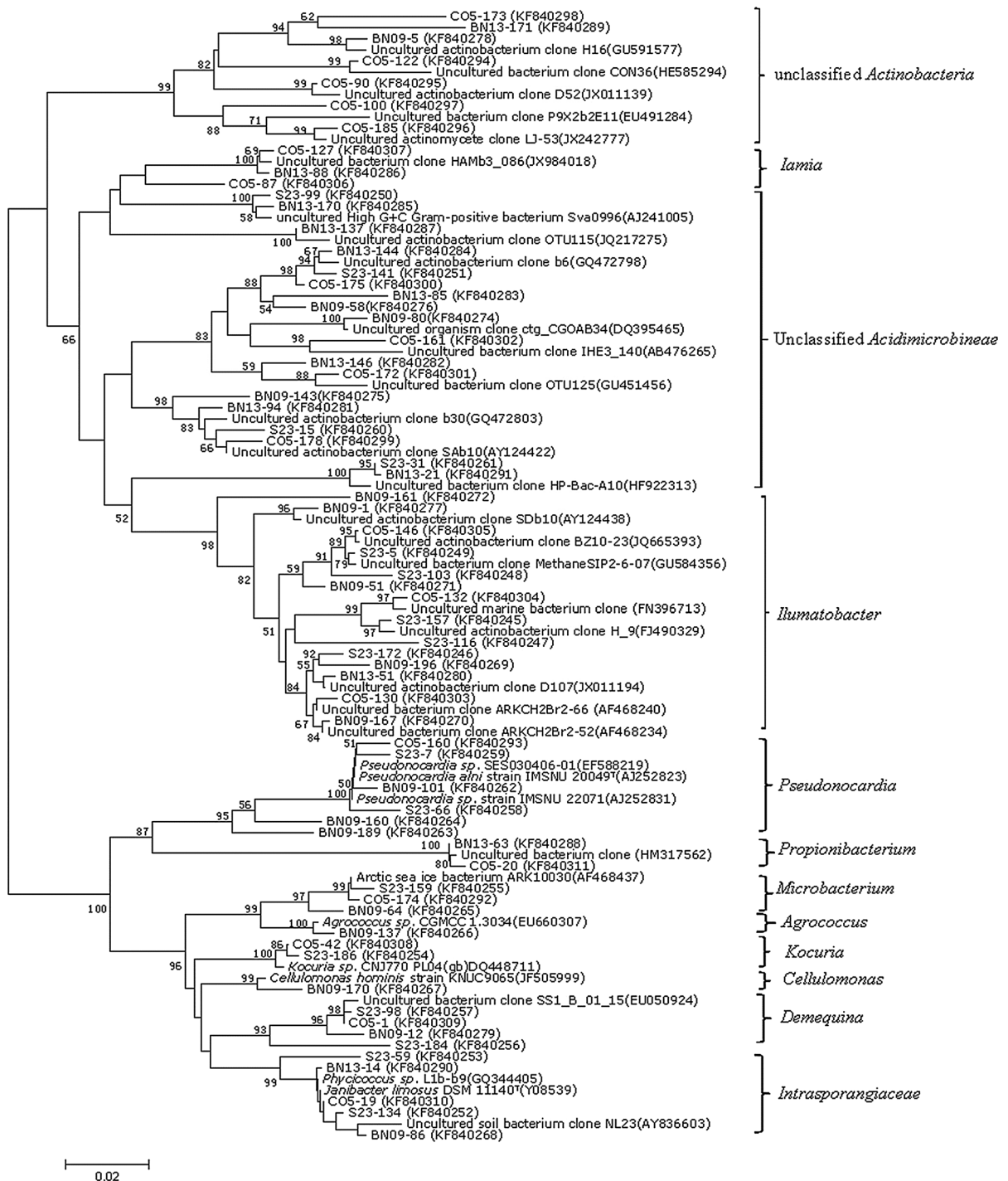


Fig. 2 Neighbor-joining tree showing the phylogenetic relationships of partial actinobacterial 16S rRNA gene sequences cloned from the studied samples to closely related sequences from the GenBank database. One representative clone within

each RFLP type is shown. Bootstrap values of >50 % (for 1,000 iterations) are shown. The scale bar corresponds to 0.02 substitutions per nucleotide position

Arctic marine sediments. The results of blast sequences analysis at the NCBI web site indicated that almost all clone sequences in the uncultured Actinobacteria were related most closely to those previously retrieved from marine environmental samples.

Based on the coverage estimates (Table 2), about 86.2–92.0 % of the diversity in the clone libraries for the four samples was sampled. The BN13 library had the highest community richness (Chao1, Table 1), followed by S23, CO5, and BN09. The 95 % CI of the Chao1 estimator overlapped among the four samples, indicating that the difference between the four libraries may be not significant. The S23 library had the highest Shannon biodiversity index, followed by BN09, BN13, and CO5. The composition and the relative abundance of each group varied among the samples (Table 2). The CO5 library was dominated by members of the suborder *Acidimicrobineae* (40 % of phylotypes and 60.7 % of sequences). Most of these belonged to the genera *Ilumatobacter* and *Iamia*. The suborder *Micrococccineae* accounted for 20 % of the phylotypes and 15.4 % of sequences. In addition, a number of sequences could not be classified to the family level in this habitat (40 % of the phylotypes, 18.4 % of sequences). In the BN13 library, 75 % of phylotypes and 93.1 % of sequences belonged to the suborder *Acidimicrobineae*, which also dominated the classified phylotypes in the BN09 library (44 % of phylotypes, 38.5 % of sequences) and S23 library (47 % of phylotypes, 50 % of sequences). The *Micrococccineae* clone sequences were the second most dominant group in the BN09 library (28 % of phylotypes, 31.9 % of sequences) and S23 library (35 % of phylotypes, 27.6 % of sequences). Although the overall distribution of taxonomic groups within the four libraries was very similar at suborder levels (Table 2), some noticeable differences were observed at the genus level. The genera *Agrococcus* and *Cellulomonas* were found exclusively in the BN09 library, and the genus *Phycococcus* was found only in the S23 library. The common genera included *Ilumatobacter* in the four libraries; *Iamia* and *Propionibacterium* in the CO5 and BN13 libraries; *Demequina*, *Microbacterium*, and *Pseudonocardia* in the CO5, BN09, and S23 libraries; *Janibacter* in the CO5, BN13, and BN09 libraries; and *Kocuria* in the CO5 and S23 libraries. Based on the LIBSHUFF analyses, the S23 clone library was significantly different from the other three clone libraries

($p < 0.025$). There was no significant difference among the CO5, BN13, and BN09 clone libraries. Such differences or similarities could be ascribed to multiple factors, such as biogeographical controls, or variations in pH, temperature, and water chemistry. Definitive resolution of these controlling factors must await future investigations.

Comparison of the actinobacterial community composition determined by culture-dependent and culture-independent approaches

It is widely understood that the use of molecular-based versus cultivation-based approaches leads to very different insights about actinobacterial diversity. These methods are complementary and should be combined, in order to reveal the true diversity of *Actinobacteria*. In this study, ten genera were identified using cultivation techniques, whereas the molecular method revealed the presence of 11 known genera and 25 potential new taxa. Only three genera (*Kocuria*, *Microbacterium*, and *Pseudonocardia*) were detected by both methods. Almost no clone sequences were identical to those of isolated actinobacteria. Seven isolated genera, such as *Actinotalea*, *Arthrobacter*, *Brachybacterium*, *Brevibacterium*, *Kytococcus*, *Micrococcus*, and *Mycobacterium* were not found in the clone libraries. One explanation is that these genera were less abundant microbes within the libraries constructed in this study. The libraries were not large enough to detect all of the actinobacteria present in low abundances, but the cultivation conditions used in this study were at least suitable for isolation of many of those microbes. DNA extraction methodology, remnant surface colonizers, and primer-biased amplification could also cause the different abundances, resulting in the underrepresentation of some genotypes from environmental samples (Baker et al. 2003). Eight actinobacteria genera (*Agrococcus*, *Cellulomonas*, *Demequina*, *Iamia*, *Ilumatobacter*, *Janibacter*, *Phycococcus*, and *Propionibacterium*), along with 25 unclassified actinobacterial groups were detected from the libraries, but were not isolated by cultivation. It is probable that this is due to limitations in current cultivation conditions and available techniques, regarding the pretreatment procedure, isolation media, and cultivation time. More innovative isolation methods and media should be developed for use in future studies.

Table 2 Ecological estimates and major group affiliations of clone sequences retrieved from four marine sediments from the Arctic region

Community	CO5	BN13	BN09	S23
Library size (no. of clones)	170	145	162	148
Coverage (%)	0.918	0.862	0.92	0.886
No. of observed OTUs	27 (20)	33 (12)	30 (18)	35 (17)
Species richness (chao1, 95 % CIs)	51.5 (33.5, 118.9)	133.0 (56.8, 453.2)	42.1 (33.4, 73.5)	63.9 (43.6, 132.5)
Shannon index (Chao & Shen, 95 % CIs)	2.657 (2.317, 2.997)	2.794 (2.261, 3.327)	2.850 (2.569, 3.131)	3.109 (2.810, 3.409)
Simpson index (MLE, 95 % CIs)	0.122 (0.064, 0.180)	0.157 (0.077, 0.237)	0.105 (0.064, 0.144)	0.086 (0.056, 0.116)
<i>Major group affiliation No. of clones (relative percentage in each actinobacterial clone library)</i>				
Actinobacteria				
Cellulomonadaceae			1 (1.1)	
Demequinaceae	15 (11.5)		6 (6.6)	9 (11.3)
Intrasporangiaceae	3 (2.3)	1 (2.3)	7 (7.7)	7 (8.7)
Microbacteriaceae	1 (0.8)		15 (16.5)	5 (6.3)
Micrococcaceae	1 (0.8)			1 (1.3)
Propionibacteriaceae	1 (0.8)	1 (2.3)		
Pseudonocardiaceae	8 (6.2)		25 (27.4)	17 (21.2)
Acidimicrobiaceae	49 (37.7)	20 (45.4)	31 (34.1)	37 (46.2)
Iamiaceae	28 (21.5)	8 (18.2)		
Unclassified Actinomycetales	1 (0.8)			
Unclassified Acidimicrobineae	2 (1.5)	13 (29.5)	4 (4.4)	3 (3.8)
Unclassified Actinobacteria	21 (16.1)	1 (2.3)	2 (2.2)	1 (1.3)
Non-Actinobacteria				
Verrucomicrobiae		1		
Candidate WS3		1		
Acidobacteria Gp22		1		
Nitrospira		1		
Planctomycetacia		1		
Gammaproteobacteria			1	
Alphaproteobacteria				1
Deltaproteobacteria	35	78	62	57
Unclassified Proteobacteria			3	1
Unclassified bacteria	5	18	5	9

Numbers in the parenthesis indicates the number of OTUs affiliated with Actinobacteria. The non-actinobacterial clone sequences were excluded for the calculation of the relative percentage of each major group in the clone libraries

Conclusions

The results of the present study indicate that a broad diversity of actinobacteria exists in the Arctic. To our knowledge, this is the first attempt to combine culture-dependent and culture-independent approaches for a

comprehensive investigation of actinobacteria communities in Arctic marine sediments. At least 18 actinobacterial genera were identified. Cultivation-independent techniques can be used to inform cultivation strategies that will improve the recovery of novel actinobacteria from marine environments. This

study lays the foundation for such work, and for further understanding of actinobacterial biodiversity and bioactivity in the Arctic.

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