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Full Length Research Paper

Culture-dependent diversity of *Actinobacteria* associated with seagrass (*Thalassia hemprichii)*

Houbo Wu[1], Wen Chen[1,2], Guanghua Wang[1]*, Shikun Dai[1,2], Danyan Zhou[1,2], Hengzhi Zhao[1,2], Yatao Guo[1,2], Yongchang Ouyang[3] and Xiang Li[1]

[1]Key Laboratory of Marine Bio-resources Sustainable Utilization (LMB-CAS), Guangdong Key Laboratory of Marine Materia Medica (LMMM-GD), South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, China.
[2]Graduate University of the Chinese Academy of Sciences, Beijing 100049, China.
[3]Guangzhou Medical University, Guangzhou 510182, People's Republic of China.

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A total of 110 culturable actinobacteria associated with seagrass (*Thalassia hemprichii*) were isolated using six different culture media, and their bioactivity potentials were analysed based on their genetic background on polyketide synthetase (PKS) and nonribosomal peptide synthetase (NRPS) gene sequences. Though the using of RFLP technique for sequencing and phylogenetic analysis, these selected 33 culturable isolates identified as belonging to ten genera of actinobacteria including *Streptomyces*, *Micromonospora*, *Saccharomonospora*, *Mycobacterium*, *Actinomycetospora*, *Nonomuraea*, *Verrucosispora*, *Nocardiopsis*, *Microbacterium* and *Glycomyces*. Four of the strains were unable to be assigned to currently known species and might be candidates of novel species. To our knowledge, this is the first report about culturable actinobacteria associated with seagrass and the isolate classified as *Verrucosispora* was first recorded to be isolated from plant. Most of the isolates harbor NRPS and PKS genes, which might indicate that these strains have great potential in production of bioactive natural compounds.

Key words: Seagrass, *Thalassia hemprichii*, actinobacteria, diversity.

INTRODUCTION

Marine associated microorganism was the spotlight during the last two decades because of the increasing demand for novel bioactive compounds (Bernan et al., 1997; Burgess et al., 1999, but still, the vast biotechnological potential of marine associated microorgansims remain mostly unexplored (Egan et al., 2008). As a special group of plants, seagrass associated microorganism had been studied through culture dependent and independent molecular approaches (Cifuentes et al., 2000; Weidner et al., 1996), with similar conclusion that seagrass was rich in bacterial diversity. Jensen et al. (2007) showed that actinobacteria was one of the most important groups in seagrass. Meanwhile, some new bacterial species were isolated from seagrass and classified as *Desulfovibrio zosterae* (Nielsen et al., 1999), *Pelagicoccus croceus* (Yoon et al., 2007), *Marinomonas balearica* and *Marinomonas pollencensis* (Espinosa et al., 2010). However, there was no report, to our knowledge, specifically investigates the community diversity of culturable actinobacteria from seagrass. Actinobacteria contributed almost half of the bioactive compounds in the Antibiotic Literature Database and rare actinobacteria were especially versatile (Lazzarini et al., 2000). Compound duplication was an increasingly important problem for today's natural product chemists.

As a successful strategy of prescreening for potential natural product drug discovery, DNA primers which target biosynthetic genes encoding for bioactive compounds were designed and applied for detecting potentially novel and useful secondary metabolites (Courtois et al., 2003;

*Corresponding author. E-mail: wgh@scsio.ac.cn.

Table 1. Composition of the 6 different media for isolation of *Actinobacteria.*

	Medium formula
M1	6 ml 100% glycerol, 0.5 g Arginine, 0.5 g K_2HPO_4, 0.25 g $MgSO_4.7H_2O$, 15 g agar and 50% of natural seawater.
M8	10 g soluble starch, 4 g yeast extract, 1 g K_2HPO_4, 1 g $MgSO_4.7H_2O$, 20 g NaCl, 15 g agar and 50% of natural seawater.
G2	1 g glucose, 0.5 g peptone, 0.3 g tryptone, 0.25 g NaCl, mixed vitamins, 15 g agar and 50% of natural seawater.
HZ	3 g trehalose, 0.5 g proline, 0.5 g $(NH_4)_2SO_4$, 0.5 g NaCl, 1 g $CaCl_2$, 0.5 g K_2HPO_4, mixed vitamins, 15 g agar and 50% of natural seawater.
G	20 g soluble starch, 1 g KNO_3, 0.5 g $MgSO_4.7H_2O$, 0.5 g K_2HPO_4, 0.02 g $CaCO_3$, 0.01 g $FeSO_4.7H_2O$, 15 g agar and 50% of natural seawater.
HC	Seagass decoction 1000 ml, 4 g tryptone, 5 g yeast extract, 15 g agar and 50% of natural seawater.

Ginolhac et al., 2004). In this study, *actinobacteria* from seagrass *Thalassia hemprichii* were isolated and classified using culture dependent approaches and their potential of producing bioactive compound such as polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) gene were also investigated.

MATERIALS AND METHODS

Seagrass sample collection

Seagrass sample was collected at Nancun Port in China in October 2010 and stored at - 20°C until further analysis. The seagrass sample was identified as *Thalassia hemprichii* by a plant taxonomist and stored at South China Sea Institute of Oceanology in Guangzhou, China.

Isolation of *actinobacteria*

The seagrass sample was washed three times using sterile seawater to remove the loosely attached bacteria and then homogenized by a stirring machine. The plant juice was diluted in series dilutions up to 10^{-1}, 10^{-2} and 10^{-3} before plating on agar medium plates (Table 1) in triplicates for each dilution. The isolation plates were incubated at 28°C for 2 to 4 weeks. Six specialized culture media adapted from Peng et al. (2007) and Sun et al. (2010) were used to isolate actinobacteria (Table 1). All culture media contain agar 15 gl^{-1}, a final concentration of 50 μgml^{-1} potassium dichromate ($K_2Cr_2O_7$), and 15 μgml^{-1} nalidixic acid to minimize the growth of fungi and non-actinobacteria eubacteria. Single colonies bearing typical actinobacteria morphology (colorful substrate mycelia, aerial mycelia, spores mass and pigment production) and Gram staining were selected and further purified on the same isolation media for purity. Purified actinobacteria were stored on 25% glycerol solution for long term storage.

DNA extraction and PCR amplification

Purifed bacterial culture was harvested from the isolation medium. Total DNA was extracted using the method of Sun et al. (2010). For 16S rDNA amplification, the genomic DNA of each isolates was amplified using bacterial universal primers 27f (5′-GAG TTT GAT CCT GGC TCA-3′) and 1500r (5′-AGA AAG GAG GTG ATC CAG CC-3′) (Woese et al., 1983). The PCR was carried out in a 25 µl PCR mixtures including Taq Premix (Takara) 12.5 µl, each of the primers (10 µM) 0.5 µl and 5% DMSO. Cycling conditions was started with an initial denaturation at 94°C for 6 min, followed with 30 cycles of 94°C for 40 s, 53°C for 40 s, and 72°C for 2 min, and finished by a final extension of 10 min at 72°C.

PKS I, PKS II and NRPS genes survey

Three sets of different oligonucleotide primers targeting PKS I, PKS II and NRPS genes were synthesized by Takara (China) followed the description by (Ayuso-Sacido and Genilloud, 2005; Metsä-Ketelä et al., 1999). The polymerase chain reaction was carried out in a 20 µl mixtures including Taq Premix 10 µl, each set of primers (10 µM) 0.5 µl and 5% DMSO with cycling conditions as follows: initial denaturation at 95°C for 5 min, 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min and a final extension of 5 min at 72°C.

16 S rDNA RFLP analysis

Amplicons were digested with restriction enzymes HhaI (TaKaRa) at 37°C for 4 h. After electrophoresis on 2% agarose gels at 50 V for 3 h, Agarose gels were photographed and analyzed using the Tanon1600 Gel Image System, version 4.00. The strains that demonstrated distinct RFLP patterns were picked out for sequencing.

Sequencing and phylogenetic analysis

Each selected 16 S rDNA amplicons was gel-purified and sequenced by BGI (Beijing) with the primer pair 27f and 1500r. All the 16S rDNA sequences were then compared with that deposited in the GenBank database using the BLAST algorithm and the EzTaxon server (www.eztaxon.org) (Chun et al., 2007). The sequences were then aligned with actinobacteria 16S rDNA data retrieved from the GenBank to generate a matrix using CLUSTALW (Thompson et al., 1997). The tree topologies were evaluated by bootstrap analyses based on 1,000 replications and phylogenetic trees were generated using the neighbor-joining method in Mega 4 (Saitou and Nei, 1987). The 16S rRNA gene sequences of representative isolates were deposited in the GenBank database with the following accession numbers: M8Z38 (JF729472), G2Z43 (JF729474), HCZ4 (JF729475), M1Z44 (JF729476), M1Z45 (JF729477), M8Z26 (JF729479), G2Z34 (JF729480), M8Z39 (JF729481), G2Z20 (JF729482) and JF806641-JF806668.

RESULTS

Diversity of actinobacteria

After a preliminary identification was made on the basis of morphological appearance and Gram staining, a total of 110 actinobacteria-like bacteria were isolated from seagrass using six culture media, some of which seems typical rare actinobacteria. However, the effectiveness of these media differed considerably with 62 isolates recovered from medium G and none from medium HZ. The BLAST results indicated that these isolates were attributed to *Streptomyces*, *Micromonospora*, *Verrucosispora*, *Saccharomonospora*, *Actinomycetospora*, *Microbacterium*, *Mycobacterium*, *Nonomuraea*, *Nocardiopsis* and *Glycomyces* (Figures 1, 2 and Table 2). Among these selected strains based on RFLP, 18 isolates belong to the dominant genera *Streptomyces*, four isolates belong to *Micromonospora*, three to *Saccharomonospora*, two to *Mycobacterium*, one isolate for each of the other six genera, respectively. Among the six different media tested, G2 had the best recoverability, with five of the total ten different genera recovered, then media M1, M8 and HC with four different genera respectively, but only *Streptomyces* recovered from media G. Isolate G2Z20 showed the closest homology with *Streptomyces varsoviensis* (97.70%), while M8Z38 had only 96.3% similarity with the closest strain *Streptomyces cacaoi* subsp in *Streptomyces.*

Among the rare actinobacterial genera, HCZ4 has 96.88% identity with a strain of *Glycomyces arizonensis*, M1Z45 shares 97.52% identity to *Nocardiopsis composta*.

Detection, distribution and analysis of NRPS, PKS-I and PKS-II

In order to take a first glimpse at whether these isolates have the potential to produce bioactive compounds, three sets of degenerate primers that target genes encoding polyketide synthases (PKS-I and II) and nonribosomal peptide synthetase (NRPS) were used to detect the potential chemical diversity of these actinobacteria (Table 2). PKS-II and NRPS sequence were amplified in almost all of the isolates, with 91.9 and 97.3% respectively. But PKS-I was detected just in 48.5% of the isolates. None of the target gene was found in strain GZ38.

DISCUSSION

Seagrass is a rich resource for culturable actinobacteria

This is the first report about culturable actinobacteria originated from seagrass, which suggested that seagrass *Thalassia hemprichii* harbored a significant amount of actinobacteria. Previously, investigation on marine culturable actinobacteria was concentrated in marine sponge (Abdelmohsen et al., 2010; Jiang et al., 2007; Khan et al., 2011; Schneemann et al., 2010; Selvin et al., 2009; Zhang et al., 2008), coral (Nithyanand and Pandian, 2009), marine sediments (Jensen et al., 2005; Maldonado et al., 2005; Pathom-aree et al., 2006; Zhao et al., 2009). The novelty of seagrass associated actinobacteria was very encouraging, four of the isolates M8Z38, HCZ4, M1Z45 and G2Z20 might be the candidate for novel species with maximum similarity of 96.3, 96.8, 97.5 and 97.7%, respectively. Current study concentrated in details of their classification and systematics. Seagrass provided a new source of rare actinobacteria. To our knowledge, this is the first time that isolates (M1Z33 and M8Z26) of the genus *Verrucosispora* were isolated from plant, the type strain *Verrucosispora gifhornensis* was first isolated from peat bog (Rheims et al., 1998), two other new species of this genera *Verrucosispora sediminis* (Dai et al., 2010) and *Verrucosispora lutea* (Liao et al., 2009) were obtained from marine sediment.

The genus *Actinomycetospora* is a new group of the order *Actinomycetales*, most of the species under this genus were from soil except for *Actinomycetospora corticicola* and *Actinomycetospora iriomotensis* which were isolated from the cortex of mangrove tree and lichen respectively (Jiang et al., 2008; Yamamura et al., 2010, 2011).

Selection of right medium is crucial for enriching culturable actinobacteria

Artificial media provided the most essential nutrition for the growth of isolates. Of the six isolation media used in this study, both the quantity and the diversity of isolates recovered were very different. Medium G without seawater was extensively applied in the isolation of actinobacteria from terrestrial environments, which recovered 62 of the total 110 isolates in this study, but it seams just suitable for recovery of *Streptomyces*. Medium G2 enable to isolate *Streptomyces*, *Micromonospora*, *Saccharomonospora*, *Mycobacterium*, *Actinomycetospora* and some other types, with *Mycobacterium* and *Actinomycetospora* as the dominant genera. *Nonomuraea* and *Nocardiopsis* were special to medium M1 and *Verrucosispora* was isolated only in medium M8. Medium HZ was not efficient for recovery of *actinobacteria*. The imitation of natural conditions was highlighted in today's microorganism isolation (Ferrari et al., 2005), Medium HC was designed based on this principle by adding seagass decoction as a supplement. As expected, four genera of *actinobacteria* (*Streptomyces*, *Glycomyces*, *Microbacteria* and *Micromonospora*) were isolated. *Microbacteria* and

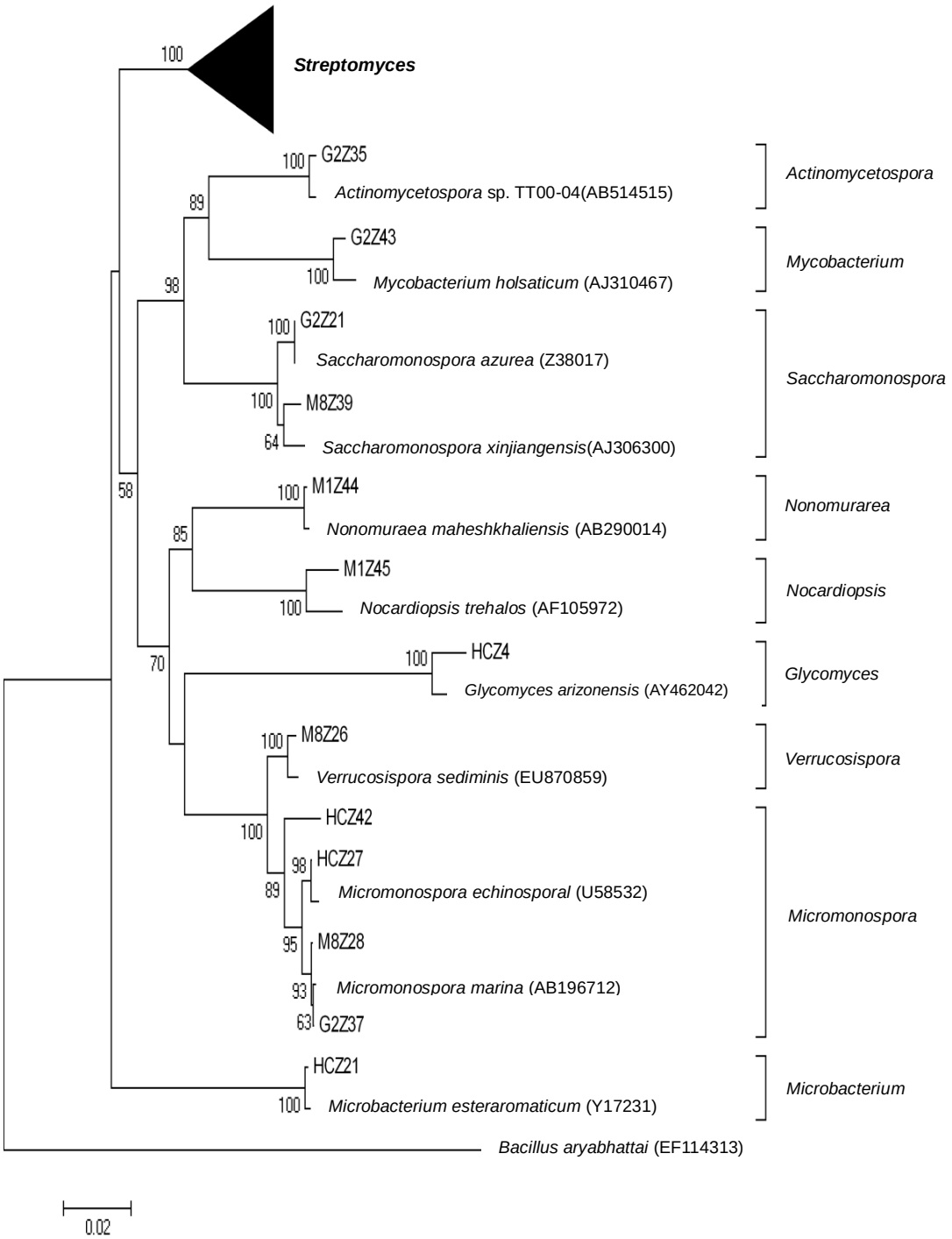

Figure 1. Neighbor-joining tree of the culturable *Actinobacteria* associated with the seagrass *Thalassia hemprichii* based on 16S rRNA gene sequences, the scale bar represents 0.02 substitutions per nucleotide position. *Bacillus aryabhattai* was used as the outgroup.

Glycomyces were only recovered in this medium. This result provide further evidence that the growth of some microorganism was environment dependent, and this characteristic should be considered in future design of medium for harvesting culturable *actinobacteria* in marine resources.

Another important consideration was the growth rate of microorganisms. Some isolates grew extremely slow and developed visible colonies in one to several months, such as isolates M8Z38, G2Z35, M1Z45, M1Z44 and M8Z28. It

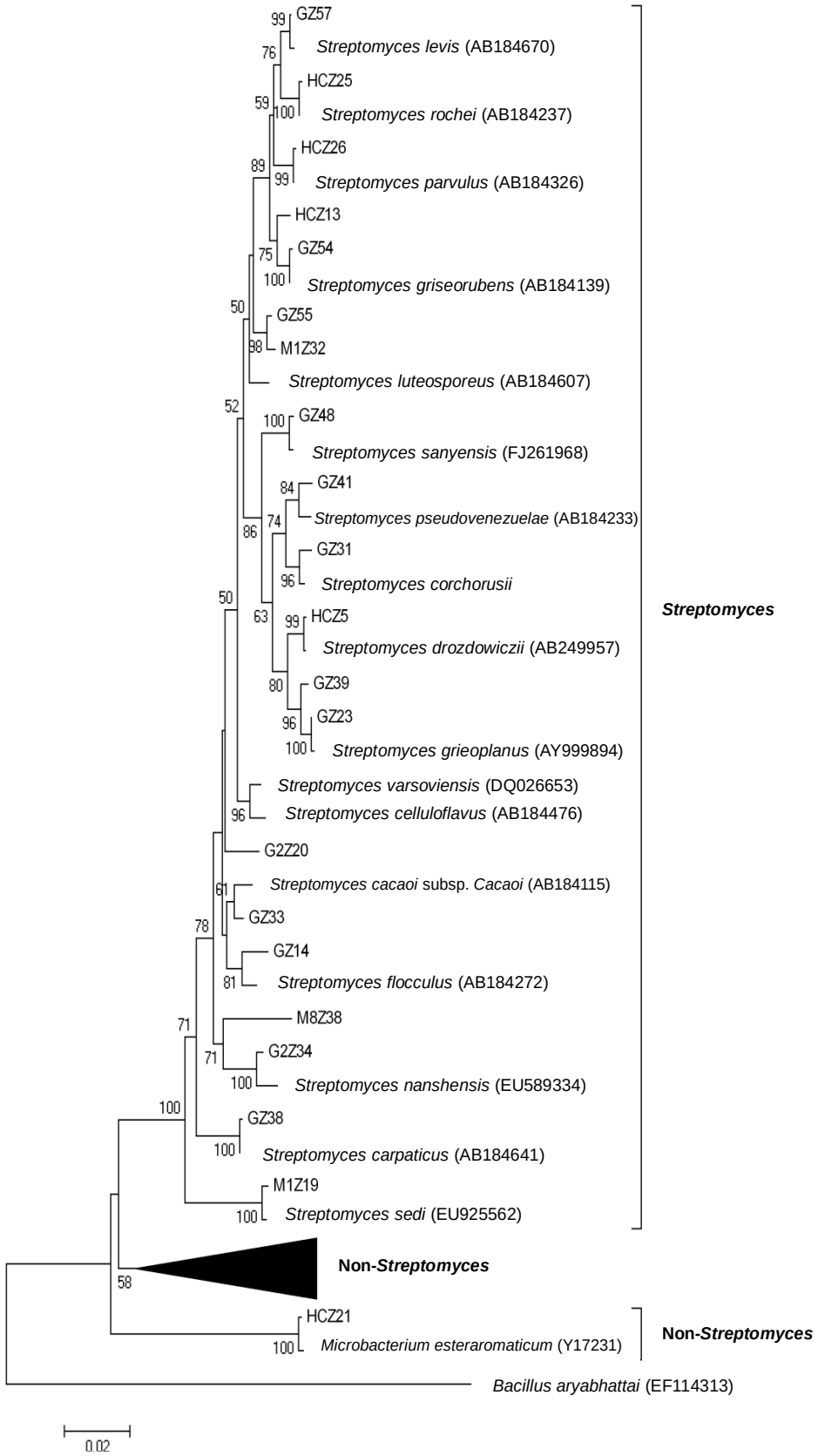

Figure 2. Neighbor-joining tree of *Steptomyces* associated with the seagrass *Thalassia hemprichii* based on 16S rRNA gene sequences, the scale bar represents 0.02 substitutions per nucleotide position. *Bacillus aryabhattai* was used as the outgroup.

Table 2. Partial *Actinomycetes* isolated from *Thalassia hemprichii*.

Strain code	The most similar species published	Identity (%)	PKS-I	PKS-II	NRPS
G2Z20*	*Streptomyces varsoviensis*	**97.70**	-	+	+
G2Z21*	*Saccharomonospora azurea*	100.00	+	+	+
G2Z34*	*Streptomyces nanshensis*	98.91	-	+	+
G2Z35*	*Actinomycetospora lutea*	99.22	-	+	+
G2Z37*	*Micromonospora aurantiaca*	99.79	-	+	+
G2Z41	*Saccharomonospora xinjiangensis*	98.44	+	+	+
G2Z43*	*Mycobacterium holsaticum*	98.70	-	+	+
M1Z19*	*Streptomyces sedi*	99.58	-	+	+
M1Z32*	*Streptomyces variabilis*	98.15	-	+	+
M1Z33	*Verrucosispora sediminis*	99.21	-	+	+
M1Z44*	*Nonomuraea maheshkhaliensis*	99.78	-	-	+
M1Z45*	*Nocardiopsis composta*	**97.52**	+	+	+
M8Z26*	*Verrucosispora sediminis*	99.21	-	+	+
M8Z28*	*Micromonospora marina*	99.78	+	-	+
M8Z38*	*Streptomyces cacaoi subsp. cacaoi*	**96.31**	-	+	+
M8Z39*	*Saccharomonospora xinjiangensis*	98.49	+	+	+
GZ8	*Streptomyces althioticus*	98.26	-	+	+
GZ14*	*Streptomyces flocculus*	98.30	+	+	+
GZ23*	*Streptomyces griseoplanus*	99.71	+	+	+
GZ28	*Streptomyces glauciniger*	99.86	-	+	+
GZ31*	*Streptomyces corchorusii*	99.50	+	+	+
GZ33*	*Streptomyces flocculus*	98.44	+	+	+
GZ38*	*Streptomyces carpaticus*	99.72	-	-	-
GZ39*	*Streptomyces celluloflavus*	99.93	-	+	+
GZ41*	*Streptomyces pseudovenezuelae*	99.14	+	+	+
GZ48*	*Streptomyces sanyensis*	99.36	-	+	+
GZ54*	*Streptomyces griseorubens*	99.79	+	+	+
GZ55*	*Streptomyces luteosporeus*	98.0	-	+	+
GZ57*	*Streptomyces levis*	99.36	+	+	+
HCZ4*	*Glycomyces arizonensis*	**96.88**	-	+	+
HCZ5*	*Streptomyces drozdowiczii*	99.65	+	+	+
HCZ13*	*S. diastaticus subsp. ardesiacus*	100	-	+	+
HCZ21*	*Microbacterium esteraromaticum*	99.0	-	+	+
HCZ25*	*Streptomyces rochei*	99.93	+	+	+
HCZ26*	*Streptomyces parvulus*	99.71	+	+	+
HCZ27*	*Micromonospora echinospora*	99.50	+	+	+
HCZ42*	*Micromonospora siamensis*	98.85	-	+	+

*Represent strains used in phylogenetic tree construction; "+" represent PCR screening for target genes is positive and "-" is that of negative.

would be an important task to design better growth conditions to promote the growth of these rare actinobacteria.

The potential of bioactive natural compound production

As a unique and important source of novel secondary metabolites (Donia and Hamann, 2003), rare actinobacteria become very precious because of the urgent demand for novel drug lead to combat increasing problems of drug resistance. They may inhabit in special environments such as marine sponge (Jiang et al., 2007; Zhang et al., 2008), medicinal plants (Zhao et al., 2011) and seagrasses. Rare actinobacteria are also aboundance in marine seagrass. Among the rare actinobacteria, most members of genus *Verrucosispora* were found to have the ability to produce bioactive compounds, for example: Abyssomicins (Riedlinger et al., 2004; Schobert and Schlenk, 2008), antitumor furan analogues of netropsin-Proximicins A, B, C and so on

(Schneider et al., 2008). PKS-II and NRPS were detected in almost all of these isolates in this study, PKS-I was amplified in nearly half of the isolates too. These results gave a glimpse of the great bioactive agent production ability of seagrass associated *actinobacteia*. In conclusion, seagrass is a great pool of *actinobacteria* resource, the diversity of culturable *actinobacteria* is abundant with representatives of ten genera present, which might be a treasure of potential bioactive chemicals need further investigation.

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