

# Culture-dependent and culture-independent diversity of *Actinobacteria* associated with the marine sponge *Hymeniacidon perleve* from the South China Sea

Wei Sun · Shikun Dai · Shumei Jiang ·  
Guanghua Wang · Guohui Liu · Houbo Wu ·  
Xiang Li

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**Abstract** In this report, the diversity of *Actinobacteria* associated with the marine sponge *Hymeniacidon perleve* collected from a remote island of the South China Sea was investigated employing classical cultivation and characterization, 16S rDNA library construction, 16S rDNA-restriction fragment length polymorphism (rDNA-RFLP) and phylogenetic analysis. A total of 184 strains were isolated using seven different media and 24 isolates were selected according to their morphological characteristics for phylogenetic analysis on the basis of their 16S rRNA gene sequences. Results showed that the 24 isolates were assigned to six genera including *Salinispora*, *Gordonia*, *Mycobacterium*, *Nocardia*, *Rhodococcus* and *Streptomyces*. This is the first report that *Salinispora* is present in a marine sponge from the South China Sea. Subsequently, 26 rDNA clones were selected from 191 clones in an *Actinobacteria*-specific 16S

rDNA library of the *H. perleve* sample, using the RFLP technique for sequencing and phylogenetic analysis. In total, 26 phlotypes were clustered in eight known genera of *Actinobacteria* including *Mycobacterium*, *Amycolatopsis*, *Arthrobacter*, *Brevibacterium*, *Microlunatus*, *Nocardioides*, *Pseudonocardia* and *Streptomyces*. This study contributes to our understanding of actinobacterial diversity in the marine sponge *H. perleve* from the South China Sea.

**Keywords** *Actinobacteria* · Diversity · Marine sponge · *Salinispora*

## Introduction

The phylum *Actinobacteria* is a major phylogenetic clade in the eubacteria and contains a wide range of Gram-positive bacteria with high G+C DNA content. *Actinobacteria* are widely distributed in terrestrial and marine habitats (Hayakawa et al. 2000; Terkina et al. 2002; Shirokikh and Merzaeva 2005; Ward and Bora 2006), and are important contributors of bioactive compounds including antibiotics, antitumor agents, enzymes and enzyme inhibitors (Lazzarini et al. 2000). In fact, over two-thirds of natural product antibiotics are produced by actinomycetes (Strohl 2003). However, as the rediscovery rate of natural products from the terrestrial *Actinobacteria* is increasing, more attention has been paid to previously untapped marine *Actinobacteria* than their terrestrial

W. Sun · S. Dai · S. Jiang · G. Wang · G. Liu ·  
H. Wu (✉) · X. Li (✉)

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, People's Republic of China  
e-mail: wuhoubu@scsio.ac.cn

X. Li  
e-mail: lixiang@scsio.ac.cn; xianglixiang@yahoo.com

W. Sun · S. Dai · G. Liu  
Graduate University of the Chinese Academy of Sciences,  
Beijing 100049, People's Republic of China

counterparts. Accordingly, an escalating number of novel metabolites derived from marine actinomycetes have been described in recent years (Lam 2006). For instance, salinosporamide A, produced by *Salinispora tropica*, is one such molecule that is currently advancing through clinical trials as an anticancer agent. Apparently, marine *Actinobacteria* provide new opportunities for natural product drug discovery.

In the past decade remarkable progresses have been made in marine actinobacterial ecology. Marine *Actinobacteria* have been recovered from various marine habitats such as the sea surface microlayer, water column, sediments, below sea subfloor, the deep biosphere (Rappe et al. 2000; Inagaki et al. 2003; Agogue et al. 2005; Maldonado et al. 2005b) and marine organisms (Webster et al. 2001; Lampert et al. 2006; Zhang et al. 2006a). Many studies on marine *Actinobacteria* were focused on marine sediments and sponges. Excitingly, some surveys on marine sediments and sponges revealed several new actinomycete taxa, some of which showed their capacity to produce novel bioactive compounds (Kim et al. 2005; Williams et al. 2005; Kwon et al. 2006; Oh et al. 2006). A wide range of *Actinobacteria* have been isolated or detected from marine sponges (Webster et al. 2001; Hentschel et al. 2002; Zhang et al. 2006a; Jiang et al. 2007). Taylor et al. (2007) summarized the known diversity of microorganisms derived from sponges in 2007 and found that the *Actinobacteria* associated with sponges were categorized in at least 28 recognized genera and 38% of their 16S rRNA gene sequences were exclusively associated with sponges. It is obvious that marine sponges can be viewed as important sources of novel *Actinobacteria*, which have great potential in exploiting new natural products.

Both culture-based and molecular-based approaches have been applied in exploring the diversity of marine sponge-derived *Actinobacteria*. Nevertheless, they provide very different views of actinobacterial diversity (Zhang et al. 2006a; Xin et al. 2008). In previous studies on *Actinobacteria* associated with the marine sponge *Hymeniacidon perleve*, cultivation methods and molecular techniques separately uncovered the existence of various *Actinobacteria* (Zhang et al. 2006a; Xin et al. 2008). In addition, one actinomycete strain isolated from the same sponge was classified as a novel *Actinoalloteichus* species (Zhang et al. 2006b). The results indicate that *H. perleve* is an ideal marine sponge for discovery of *Actinobacteria*. However, the marine

*H. perleve* isolates previously investigated populate the Yellow Sea extending across temperate coast region with significant human influence. It is of interest to investigate the same sponge species inhabiting at a tropically remote island of the South China Sea with less artificial interference. The aim of this study is to explore the diversity of *Actinobacteria* associated with *H. perleve* from a remote island in the South China Sea.

## Materials and methods

### Sponge collection

Specimens of the marine sponge *H. perleve* were collected by scuba diving in May 2008 at a depth of 8 to 10 m from Yongxing Island (16°50'N; 112°20'E), Xisha Islands, the South China Sea. The samples were kept in fresh seawater on ice and then stored at -20°C in the laboratory.

### Isolation of sponge-associated *Actinobacteria*

Seven types of media were prepared for the isolation of *Actinobacteria* (Table 1). All media contained Difco Bacto agar (15 g l<sup>-1</sup>) and were adjusted to pH 7.2. All media were supplemented with a final concentration of 50 µg ml<sup>-1</sup> potassium dichromate (K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>) to inhibit fungal growth and 15 µg ml<sup>-1</sup> nalidixic acid to inhibit many fast-growing Gram-negative bacteria. To facilitate the growth of sponge-associated *Actinobacteria*, all media were supplemented with 2% sponge water extract. The water extract of sponge was prepared by placing 20 g tissue in 200 ml sterile distilled H<sub>2</sub>O for 4 h and the water was filter sterilized (0.22 µm pore size) prior to addition to media.

Sponge specimens were rinsed three times in sterile seawater to remove transient and loosely attached bacteria and then thoroughly homogenized in a homogenizer. A 10-fold dilution series of sponge homogenate was made and aliquots (100 µl) were plated in triplicate on agar plates. The inoculated plates were incubated at 28°C for 4–8 weeks.

### Strain culture, DNA extraction, 16S rRNA gene amplification and sequencing

Actinomycete colonies were preliminarily recognized by the presence of branching, vegetative filaments

**Table 1** Composition of the 7 different media for isolation of *Actinobacteria* from the marine sponge *H. perleve*

Medium formula
M1 6 ml 100% glycerol, 1 g arginine, 1 g K <sub>2</sub> HPO <sub>4</sub> , 0.5 g MgSO <sub>4</sub> ·7H <sub>2</sub> O, 15 g agar and 1 l of natural seawater
M2 10 g soluble starch, 1 g NaCl, 1 g K <sub>2</sub> HPO <sub>4</sub> , 1 g MgSO <sub>4</sub> ·7H <sub>2</sub> O, 2 g (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 2 g CaCO <sub>3</sub> , 1 ml trace element solution, 15 g agar and 1 l of natural seawater
M3 10 g soluble starch, 4 g yeast extract, 2 g peptone, 15 g agar and 1 l of natural seawater
M4 1 g glucose, 0.5 g peptone, 0.3 g tryptone, 0.5 g NaCl, mixed Vitamins, 15 g agar and 1 l of natural seawater
M5 2 g soluble starch, 0.5 g KNO <sub>3</sub> , 1.7 g KCl, 0.5 g MgSO <sub>4</sub> ·7H <sub>2</sub> O, 0.5 g Na <sub>2</sub> HPO <sub>4</sub> , 0.02 g CaCO <sub>3</sub> , 0.01 g FeSO <sub>4</sub> ·7H <sub>2</sub> O, mixed Vitamins, 15 g agar and 1 l of natural seawater
M6 2 g myo-inositol, 0.5 g tryptophane, 0.8 g NaCl, 1 g K <sub>2</sub> HPO <sub>4</sub> , 0.5 g MgSO <sub>4</sub> ·7H <sub>2</sub> O, 0.1 g CaCO <sub>3</sub> , mixed vitamins, 1 ml trace element solution, 15 g agar and 1 l of natural seawater
M7 5 g trehalose, 1 g proline, 1 g (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 1 g NaCl, 2 g CaCl <sub>2</sub> , 1 g K <sub>2</sub> HPO <sub>4</sub> , 1 g MgSO <sub>4</sub> ·7H <sub>2</sub> O, mixed vitamins, 15 g agar and 1 l of natural seawater
Trace element solution 0.1 g FeSO <sub>4</sub> ·7H <sub>2</sub> O, 0.1 g ZnSO <sub>4</sub> ·7H <sub>2</sub> O, 0.1 g MnCl <sub>2</sub> ·4H <sub>2</sub> O and 100 ml H <sub>2</sub> O
Mixed Vitamins VB1, VB2, VB3, VB5, VB6, myo-inositol and VH

and the formation of tough, leathery colonies that adhered to the agar surface. Morphologically diverse candidate actinomycetes were transferred onto solid media until pure cultures were obtained.

The isolates bearing distinct colony morphotypes on the agar plates were selected and inoculated into the same liquid media on which the colonies were initially isolated. The cells were collected by centrifugation and stored in 20% glycerol at  $-20^{\circ}\text{C}$  Freezer. The genomic DNA was extracted using the following method. The collected cells were suspended in 400  $\mu\text{l}$  of TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0). 50  $\mu\text{l}$  of 1% (wt/vol) lysozyme was added and the solution incubated at  $37^{\circ}\text{C}$  for 1.5 h. 50  $\mu\text{l}$  of 0.5 M EDTA, 50  $\mu\text{l}$  of 1% (wt/vol) SDS, 10  $\mu\text{l}$  of 0.1% (wt/vol) proteinase K were added and the solution incubated at  $55^{\circ}\text{C}$  for 3 h. 1/2 volume of 7.5 M ammonium acetate was added and mixed gently. The extract was centrifuged at 12,000 for 15 min and the supernatant transferred to a new tube. An equal volume of isopropanol was added and the solution placed at  $-20^{\circ}\text{C}$  for 30 min to precipitate the DNA. The preparation was centrifuged at 12,000 for 10 min to collect the DNA. The DNA pellet was washed twice with 70% ethanol and then air-dried for 10 min. The DNA pellet was resuspended in 50  $\mu\text{l}$  TE buffer. Universal bacterial primers 27f (5'-GAGTTTGATCCTGGCTCAG-3') and 1500r (5'-AGAAAGGAGGTGATCCAGCC-3') were used to amplify 16S rRNA genes (Woese et al. 1983). The PCR was carried out in a 20  $\mu\text{l}$  volume. PCR mixtures included Taq Premix 10  $\mu\text{l}$ , 1  $\mu\text{l}$  27f

(10  $\mu\text{M}$ ), 1  $\mu\text{l}$  1500r (10  $\mu\text{M}$ ) and 5% DMSO. Cycling conditions were as follows: initial denaturation at  $95^{\circ}\text{C}$  for 3 min, 30 cycles of  $94^{\circ}\text{C}$  for 30 s,  $54^{\circ}\text{C}$  for 40 s, and  $72^{\circ}\text{C}$  for 2 min, and a final extension of 10 min at  $72^{\circ}\text{C}$ . The purified PCR products were sequenced by Guang Zhou Jing Rui Biotech Co., LTD.

Culture-independent DNA extraction, PCR and partial 16S rRNA gene cloning

Sponge-associated bacteria were collected using differential centrifugation technology. Sponge tissue (10 g) was rinsed with sterile seawater to remove the loosely attached bacteria and then homogenized in a homogenizer containing 50 ml of sterile seawater. Five milliliter of the homogenate was aspirated into a centrifuge tube for an initial centrifugation at 250 g for 1 min. The supernatant was taken gently to a new tube for a centrifugation at 8,000g for 20 min. The collected bacteria cells were suspended in TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0). The total genomic DNA was extracted using the method described above.

Actinobacterial 16S rRNA genes were amplified by a nested PCR method. In the first PCR a 1:10 dilution of the total genomic DNA was used as a template. Universal bacterial primers 27f and 1500r were used to amplify nearly full-length 16S rRNA genes. The first PCR was carried out using a PTC200 (Bio-RAD) thermocycler in a 50  $\mu\text{l}$  volume. PCR mixtures included Taq Premix (Takara, China) 25  $\mu\text{l}$ ,

1.25  $\mu$ l 27f (10  $\mu$ M), 1.25  $\mu$ l 1500r (10  $\mu$ M) and 5% DMSO. Cycling conditions were as follows: initial denaturation at 95°C for 3 min, 30 cycles of 94°C for 30 s, 54°C for 40 s, and 72°C for 2 min, and a final extension of 10 min at 72°C. In the second PCR a 1:50 dilution of the first PCR product was used as template. *Actinobacteria*-specific primers S-C-Act-0235-a-S-20 (5'-CGCGGCCTATCAGCTTGTTG-3') and S-C-Act-0878-a-A-19 (5'-CCGTACTCCCCAGGCGGGG-3') were used to amplify an approximately 640 bp stretch of the actinobacterial 16S rRNA genes (Stach et al. 2003). The second PCR was performed in a 20  $\mu$ l volume. PCR mixtures consisted of Taq Premix 10  $\mu$ l, 1  $\mu$ l S-C-Act-0235-a-S-20 (10  $\mu$ M), 1  $\mu$ l S-C-Act-0878-a-A-19 (10  $\mu$ M) and 5% DMSO. Cycling conditions were as follows: initial denaturation at 95°C for 4 min, 30 cycles of 95°C for 45 s, 68°C for 45 s, and 72°C for 1 min, and a final extension of 5 min at 72°C. The products were combined and purified by electrophoresis in a 1% (wt/vol) agarose gel. The band of approximately 640 bp was excised and recovered using Agarose Gel DNA Purification Kit (TaKaRa). Purified PCR products were cloned into the pMD18-T vector and transformed into CaCl<sub>2</sub>-competent *Escherichia coli* DH5 $\alpha$ . Individual clones were transferred onto LB agar plates (each containing 50  $\mu$ g of ampicillin/ml) and grown overnight at 37°C. Individual plasmid inserts were amplified by direct PCR of clone cell cultures with M13 primers M4 (GTTTTCCAGTCACGAC) and RV (CAGGAAACAGCTATGAC).

#### Restriction fragment length polymorphism (RFLP) and sequencing

The amplification products of all the positive clones were digested with the restriction enzyme *Hha*I (TaKaRa) for 4 h at 37°C and then electrophoresed in a 2% agarose gel at 50 V for 2 h. Agarose gels were photographed and analyzed using the Tanon1600 Gel Image System, version 4.00. The clones that demonstrated distinct RFLP patterns were sequenced by Guang Zhou Jing Rui Biotech Co., LTD using the primer RV.

#### Phylogenetic analysis

The sequence data were proofread using Chromas, version 1.62 (Technelysium) and examined for the

formation of chimeras using the program CHECK CHIMERA in RDP II (<http://rdp8.cme.msu.edu/>). All the sequences were compared to 16S rRNA genes in the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm (<http://www.ncbi.nlm.nih.gov/>). Phylogenetic analyses were performed with program MEGA 4.1 (Molecular Evolutionary Genetics Analysis, Version 4.1) (Tamura et al. 2007). The tree topologies were evaluated by bootstrap analyses based on 1,000 replicates and phylogenetic trees were generated using the neighbor-joining method (Saitou and Nei 1987). Only bootstrap values of more than 50% were shown on the phylogenetic trees.

#### 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 numbers: GQ504216-504239 and GQ504240-504263.

## Results

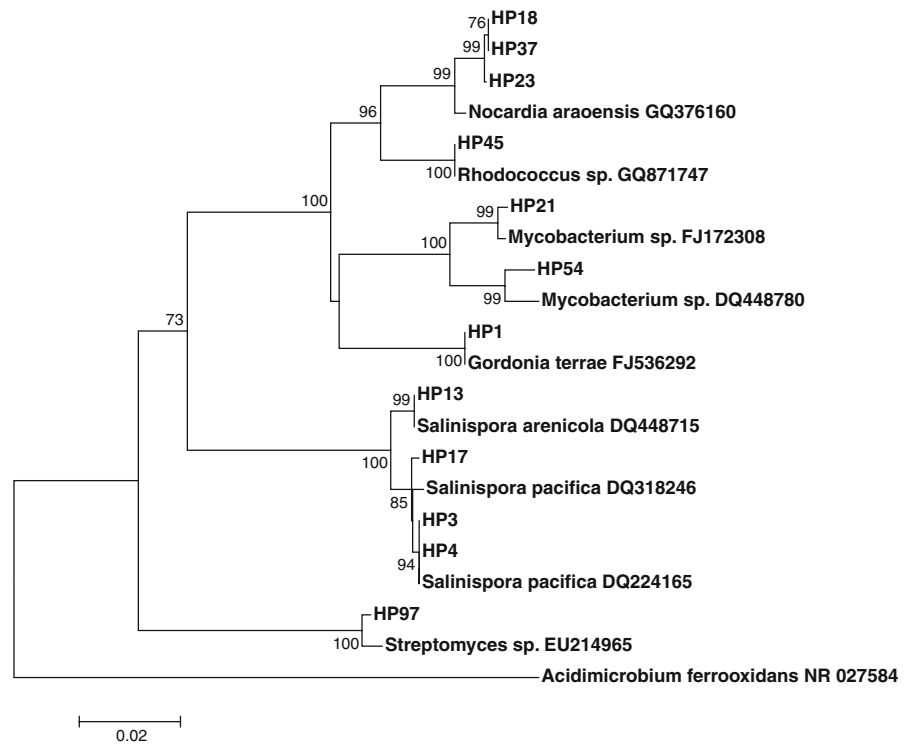
### Culture-dependent diversity of *Actinobacteria*

Seven media were employed for the isolation of *Actinobacteria*. However, the effectiveness of the different media differed considerably. Medium M1 produced the most actinomycete colonies and the highest morphological diversity, followed by M5. Media M2, M4 and M6 yielded some bacterial colonies but most colonies didn't exhibit actinomycete-like features. No actinomycete-like bacterial colonies were recovered on M3, and no colonies were recovered on M7.

A total of 184 putative actinobacterial isolates were obtained from the isolation plates and transferred to new plates for purification. Based on visible examination of the growth characteristics, 28 representative isolates were selected for further analysis.

The 16S rRNA genes of the 28 isolates were sequenced. After BLAST analysis, it was found that 24 isolates could be assigned to *Actinobacteria* whereas the other 4 isolates shared close homology with *Proteobacteria*. The phylogenetic analysis indicated that the culturable actinomycetes in *H. perleve* were clustered with members of *Salinispora*, *Nocardia*,

**Fig. 1** Phylogenetic analysis of the culturable *Actinobacteria* associated with the marine sponge *H. perleve*. The neighbor-joining tree is based on 1,400 bp of 16S rRNA gene sequence and includes the 12 strains for which unique sequence was obtained. The scale bar represents 0.02 substitution per nucleotide position. *Acidimicrobium ferrooxidans* (NR\_027548) was used as the outgroup



*Rhodococcus*, *Mycobacterium*, *Gordonia* and *Streptomyces* (Fig. 1). The majority of the isolates belonged to *Salinispora*. Seven isolates were closely related to *Salinispora pacifica* strain CNS-237 (DQ318246), with 99.8% 16S rRNA gene sequence identity. CNS-237 was originally isolated from Palau and is known to produce a series of unique pyrones characterized as salinispyrone A (Jensen et al. 2007). Four isolates showed 100% homology to *S. pacifica* strain CNH732 RS00 (DQ224165), which was originally isolated from the Red Sea and produces cyanosporaside A (Jensen and Mafnas 2006; Jensen et al. 2007). Two isolates shared 100% homology with *Salinispora arenicola* (DQ448715) isolated from Palau. The second dominant group in the culturable actinomycetes was *Nocardia*. Four isolates fell into this genus, three of which shared 98.7–98.9% 16S rRNA gene sequence homology with *Nocardia araoensis* W9705 (GQ376160). In addition, three isolates were closely related to *Rhodococcus* sp. SCSIO 00026 (GQ871747), which was isolated from marine sediment in the South China Sea. Two isolates were assigned to *Mycobacterium*, one appearing closely related to *Mycobacterium* sp. ATCC 25791 (FJ172308) and the other was

distantly related to *Mycobacterium* sp. CNJ879 PL04 (DQ448780) from marine sediment. One isolate showed 99.9% 16S rRNA gene sequence identity to *Gordonia terrae* strain IFM 0412 (FJ536292) and one isolate was closely related to *Streptomyces* sp. CNS-669\_SD06 (EU214965), which was isolated from marine sediment off the coast of California.

Culture-independent diversity of *Actinobacteria*

All the clones were PCR-amplified using M13 primers M4 and RV. A total of 191 positive clones were obtained and the amplification products were preliminarily characterized by RFLP analysis. Twenty-nine clones representing 29 different RFLP patterns were selected for sequencing. All the sequences were analyzed using the CHIMERA\_CHECK algorithm of the RDP and three chimeric sequences were eliminated. The remaining 26 partial 16S rRNA gene sequences representative of the operational taxonomic units (OTUs) were submitted to the GenBank for BLAST search to obtain the closest match for preliminary taxonomic assignments (Table 2). Of the 26 OTUs, 3 OTUs were most related to unidentified



**Table 2** Sequence analysis of the culture-independent *Actinobacteria* clones from the marine sponge *H. perleve*, based on partial 16S rRNA gene sequencing

Representative clone and GenBank acc. no.	Number of clones	GenBank acc. no. of the nearest species and similarity	Subgroup
Clone H3 (GQ504240)	87	AM936702 99%	<i>Mycobacterium</i>
Clone H4 (GQ504241)	39	AM936702 98%	<i>Mycobacterium</i>
Clone H12 (GQ504242)	6	AM936702 99%	<i>Mycobacterium</i>
Clone H25 (GQ504243)	1	AM936702 98%	<i>Mycobacterium</i>
Clone H48 (GQ504244)	3	AM936702 97%	<i>Mycobacterium</i>
Clone H56 (GQ504245)	1	DQ448781 99%	<i>Mycobacterium</i>
Clone H6 (GQ504246)	24	FJ529703 99%	<i>Amycolatopsis</i>
Clone H30 (GQ504247)	1	FJ529703 98%	<i>Amycolatopsis</i>
Clone H111 (GQ504248)	1	FJ529703 98%	<i>Amycolatopsis</i>
Clone H46 (GQ504249)	2	AB327252 97%	<i>Amycolatopsis</i>
Clone H122 (GQ504250)	1	AB327252 96%	<i>Amycolatopsis</i>
Clone H140 (GQ504251)	1	AB327252 97%	<i>Amycolatopsis</i>
Clone H23 (GQ504252)	4	EU086805 99%	<i>Arthrobacter</i>
Clone H138 (GQ504253)	2	EU086805 97%	<i>Arthrobacter</i>
Clone H150 (GQ504254)	1	EU086805 99%	<i>Arthrobacter</i>
Clone H45 (GQ504255)	2	FJ218364 99%	<i>Brevibacterium</i>
Clone H15 (GQ504256)	1	EU741106 97%	<i>Microclunatus</i>
Clone H178 (GQ504257)	1	DQ188729 100%	<i>Nocardioidea</i>
Clone H33 (GQ504258)	1	FJ532384 97%	<i>Pseudonocardia</i>
Clone H127 (GQ504259)	1	FJ999670 99%	<i>Streptomyces</i>
Clone H5 (GQ504260)	1	EU532580 100%	Unidentified <i>Actinobacteria</i>
Clone H141 (GQ504261)	1	DQ269047 98%	Unidentified <i>Actinobacteria</i>
Clone H171 (GQ504262)	1	AY711771 98%	Unidentified <i>Actinobacteria</i>
Clone H90 (GQ504263)	2	EF693544 96%	Unidentified bacterium
Clone H123	2	DQ990355 93%	<i>Planctomycete</i>
Clone H59	1	EF092213 96%	<i>Delta-proteobacteria</i>

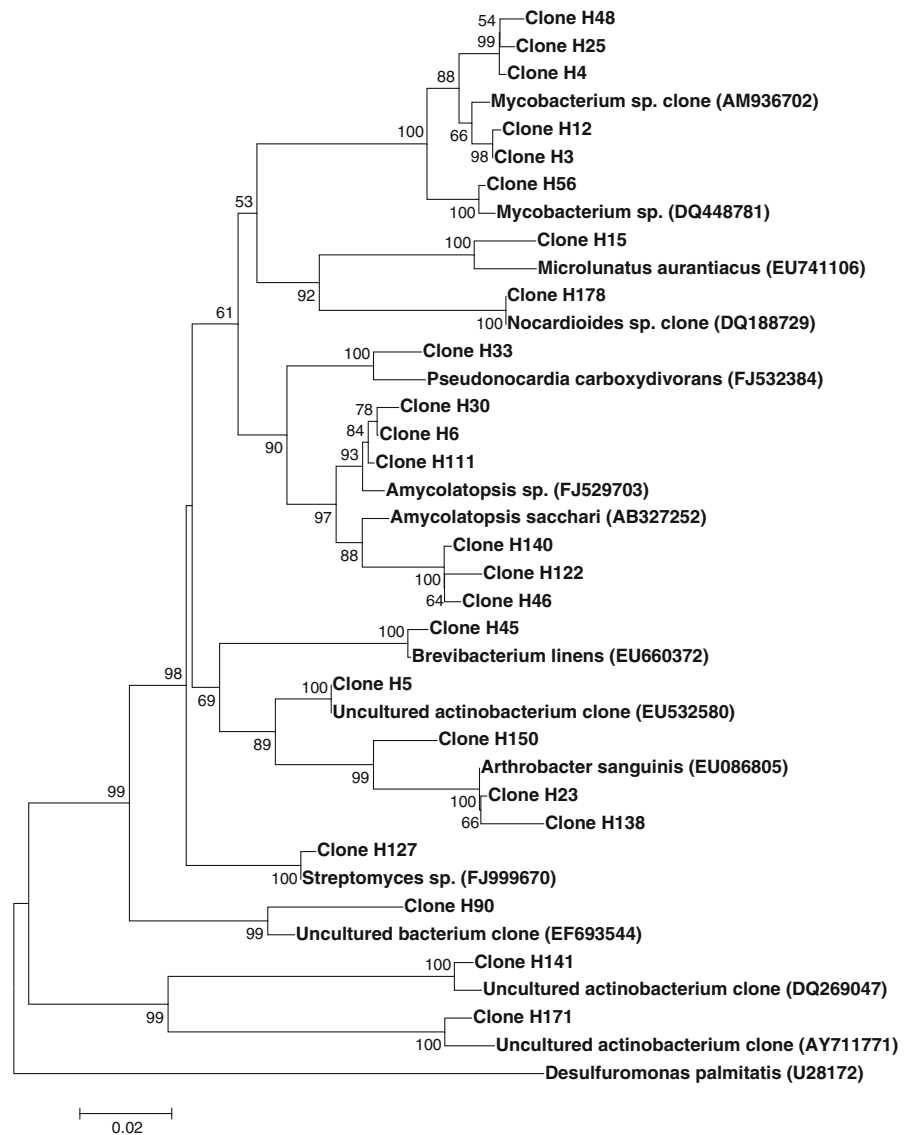
*Actinobacteria*, one OTU (Clone H90) was closest to an unidentified bacterium and the other 22 OTUs could be grouped into specific taxa, in which 20 OTUs were affiliated with *Actinobacteria*. The remaining two OTUs were matched to *Planctomycete* and *Proteobacteria*, respectively. Twenty OTUs representing *Actinobacteria* all fell into the subclass *Actinobacteridae*, in which six OTUs were grouped in the genus *Mycobacterium*, six OTUs were members in the genus *Amycolatopsis*, three OTUs were categorized in the genus *Arthrobacter* and the other five OTUs were matched to representatives of the genera *Brevibacterium*, *Microclunatus*, *Nocardioidea*, *Pseudonocardia* and *Streptomyces*. The dominant *Actinobacteria* in *H. perleve* residing in Yongxing Island of the South China Sea was *Mycobacterium*, accounting for 72%

of the clones. The second abundant group was *Amycolatopsis*, representing 16% of the clones. The results demonstrated that there is a high diversity of actinobacterial phylotypes associated with the marine sponge *H. perleve* from the South China Sea and revealed the predominant group in the actinobacterial community.

The phylogenetic analysis showed Clone H90, the nearest relative of which was an unidentified bacterium, could also be clustered in *Actinobacteria* (Fig. 2). Therefore, from the obtained 26 OTUs, a total of 24 OTUs represented a wide range of *Actinobacteria*, in which 14 OTUs shared the closest sequence homology with culturable *Actinobacteria*, while 10 OTUs were closely related to uncultured microorganisms.

The most abundant group in the actinobacterial community was *Mycobacterium*. Six OTUs represented

**Fig. 2** Phylogenetic analysis of the culture-independent *Actinobacteria* clones from the marine sponge *H. perleve*. The neighbor-joining tree is based on 643 bp of 16S rRNA gene sequence and includes the 24 clones for which unique sequence was obtained. The scale bar represents 0.02 substitution per nucleotide position. *Desulfuromonas palmitatis* (U28172) was used as the outgroup



72% of the clones. Five clones (H3, H4, H12, H25 and H48) showed close sequence identity to an uncultured *Mycobacterium* sp. clone (AM936702). The clone was recovered from a pilot-scale bioremediation process of a hydrocarbon-contaminated soil. One clone (H56) showed 99% sequence identity to *Mycobacterium* sp. CNJ881 PL04 (DQ448781) isolated from marine sediment. The next most abundant group was *Amycolatopsis*. A total of six OTUs accounted for 16% of the clones. Three clones (H6, H30 and H111) were most closely related to *Amycolatopsis* sp. CPCC202698 (FJ529703), which was isolated from soil collected at an altitude

above 2,000 m. Three clones (H46, H122 and H140) showed close 16S rRNA gene sequence homology to that of *Amycolatopsis sacchari* strain NBRC 100339 (AB327252), the type strain of *A. sacchari*. The other OTUs, which added up to 12% of the clones, represented the minor groups in the actinobacterial community. The sequences of three clones (H23, H138 and H150) were most closely related to *Arthrobacter sanguinis* strain 741 (EU086805), the type strain of *A. sanguinis*. Five clones were matched to the genera *Brevibacterium*, *Microlunatus*, *Nocardioides*, *Pseudonocardia* and *Streptomyces*, respectively. Clone H45 was most related

to *Brevibacterium linens* strain S3-6 (FJ218364), which was isolated from the surface seawater from Atlantic Ocean. The closest relative of Clone H15 was *Micro-lunatus aurantiacus* strain 13629F (EU741106), which was isolated from marine sediments. Clone H178 showed 100% 16S rRNA gene sequence homology to an uncultured *Nocardioides* sp. clone (DQ188729). Clone H33 was distantly related to *Pseudonocardia carboxyd-ivorans* strain W14-3 (FJ532384) from soil, sharing 97% 16S rRNA gene sequence homology. Clone H127 was closely related to *Streptomyces* sp. AZS17 (FJ999670), which was isolated from the marine sponge *Hymeniacidon* sp. collected in the East China Sea. The remaining 4 clones corresponded to some unidentified *Actinobacteria*, among which Clone H141 and H171 were deeply branched and may represent new lineages.

## Discussion

### Cultivation approach and the discovery of *Salinispora* from *H. perleve*

Marine sponges are the lowest marine invertebrates and harbor a variety of microorganisms in their tissues (Wang 2006). To better understand the diversity of culturable actinomycetes associated with marine sponges, it is critical to select appropriate isolation media and culture conditions. In order to maximize the recovery rates for actinomycetes, seven types of media were used in this study. Three media (M1–M3) were used to isolate common actinomycetes and four media (M4–M7) were targeted for rare actinomycetes. In addition, it is reported that the addition of aqueous sponge extract to media can result in an increase in the number of novel cultivated morphotypes (Webster et al. 2001). Therefore, the aqueous sponge extract was included in each medium in this study. M1 medium (glycerol arginine agar medium) yielded the highest diversity of cultured actinomycetes, with representatives of five genera (*Salinispora*, *Gordonia*, *Mycobacterium*, *Nocardia* and *Rhodococcus*) recovered. Similar results were observed in a study on the culturable actinomycetes in *H. perleve* from the Yellow Sea (Zhang et al. 2006a). This medium was also the best among 12 media tested, which further confirms that glycerol arginine agar medium is highly effective for the isolation of actinomycetes from marine sponges.

One of the significant findings in this study is the isolation of representatives of the genus *Salinispora* from *H. perleve*. *Salinispora* is the first obligate marine genus in the order *Actinomycetales* and members are widely distributed in tropical and subtropical marine sediments (Jensen et al. 2005; Jensen and Mafnas 2006). So far three *Salinispora* species have been identified and they are respectively *Salinispora tropica*, *S. arenicola* and *S. pacifica* (Maldonado et al. 2005a; Oh et al. 2006). To our knowledge, prior to this study the only sponge from which *Salinispora* was recovered was the marine sponge *Pseudoceratina clavata* from the Great Barrier Reef (Kim et al. 2005). Isolates related to *S. pacifica* and *S. arenicola* were successfully cultivated although *Salinispora* was not detected by the culture-independent nested PCR method. This is the first report to recover *Salinispora* from marine sponge inhabiting the South China Sea. The abundance and diversity of these *Salinispora* strains in marine sponges are possibly not lower than those in marine sediments. It is known that the three *Salinispora* species all possess the ability to produce important secondary metabolites. *S. tropica* can produce salinosporamide A showing cytotoxic activity and sporolide A (Williams et al. 2005). *S. pacifica* can produce a novel metabolite cyanosporaside A (Oh et al. 2006). Recently, some *Salinispora* strains isolated from Great Barrier Reef sponges have been shown to produce rifamycins (Kim et al. 2006). The strains of *S. pacifica* and *S. arenicola* in our study share 99–100% 16S rRNA gene sequence identity with their closest relatives. It is reported that the different intraclade phylogenotypes of *S. pacifica* may produce different secondary metabolites of unique chemical structures (Jensen et al. 2007). Therefore, it is likely that the strains from the marine sponge *H. perleve* in the South China Sea have great potential for natural product search and discovery.

### Molecular approaches

Nested PCR methods have proven effective for assessing uncultured *Actinobacteria* in various environmental samples (Rheims and Stackebrandt 1999). The *Actinobacteria*-specific primers designed by Stach et al. (S-C-Act-0235-a-S-20 and S-C-Act-0878-a-A-19) have high specificity and low coverage and can detect a high diversity in a relatively small clone library



(Stach et al. 2003). In a study on the diversity of *Actinobacteria* associated with *H. perleve* from the Yellow Sea, the use of the primers revealed a high actinobacterial diversity (Xin et al. 2008). This set of primers was applied in our study. To this end, 24 out of 26 representative clones were proved to be of actinobacterial origin. A total of eight actinobacterial groups (*Mycobacterium*, *Amycolatopsis*, *Arthrobacter*, *Brevibacterium*, *Microlunatus*, *Nocardioides*, *Pseudonocardia* and *Streptomyces*) were finally identified. In addition, four other OTUs which could not be identified by the phylogenetic analysis may represent some other known or unknown actinobacterial groups present in *H. perleve*. Our study further supports the proposition that the nested PCR method, together with 16S rRNA gene library construction, is effective for the detection of uncultured *Actinobacteria*.

#### Comparison between the culture-independent and the culture-dependent diversity

It is widely accepted that molecular-based and cultivation-based approaches lead to very different insights into actinobacterial diversity (Zhang et al. 2006a; Xin et al. 2008). They are complementary and should be combined to reveal the complete diversity of *Actinobacteria*. In our study, the molecular method uncovered eight actinobacterial genera and the cultivation techniques recovered six actinobacterial genera. Only two genera (*Mycobacterium* and *Streptomyces*) were detected by both approaches. Molecular technique revealed that *Mycobacterium* spp. were the dominant *Actinobacteria* within *H. perleve*. However, *Mycobacterium* was only a small component of the cultured actinomycetes. Conversely, not only the molecular approach but also the cultivation methods revealed *Streptomyces* was of low-abundance among the *Actinobacteria*. Only one out of 191 clones was assigned to *Streptomyces*. Similarly, only one strain of 184 isolates belonged to *Streptomyces*. The results show that *Streptomyces* is a minor group in *H. perleve*. The six genera (*Amycolatopsis*, *Arthrobacter*, *Brevibacterium*, *Microlunatus*, *Nocardioides* and *Pseudonocardia*) detected by molecular method were not isolated by cultivation in this study. Apparently, this is due to the limitations of the cultivation conditions. Selecting genus-specific/bias isolation media might allow the growth of these actinomycetes. The four genera (*Salinispora*, *Gordonia*, *Nocardia* and

*Rhodococcus*) cultivated successfully on the media were not detected in our clone library. Since the primers showed a perfect match with every one of these four genera we supposed the explanation might be as follows. First, the amplicon library we constructed is not large enough to detect all the low-abundance *Actinobacteria*. Second, when actinomycetes form spores, their DNA cannot be extracted from spores by the chemical lysis method and so it is unsurprising that they cannot be detected. The results indicate that the inherent limitation of molecular techniques can be partly made up by culture-based method and vice versa. It is essential to apply both culture-independent and culture-dependent approaches to describe the actinobacterial diversity.

Interestingly, we isolated some *Salinispora* strains and detected some *Amycolatopsis* sequences. Although in phylogenetic analyses using 16S rRNA sequences the genera *Salinispora* and *Amycolatopsis* are not closely related, they both produce rifamycins. Rifamycins are now known to be produced by the soil bacterium *Amycolatopsis mediterranei* and marine *Salinispora* strains from the marine sponge *Pseudoceratina clavata*. The representatives of these two genera present in the marine sponge *H. perleve* are a potential source of new rifamycin synthesis genes.

#### Biogeography of *Actinobacteria* associated with marine sponges from the South China Sea

Located in the west of the Pacific and extending across the tropical and subtropical regions, the South China Sea is the largest marginal sea in the world. Yongxing Island is located in the central Xisha Islands and is the biggest island in the South China Sea. Being an island far away from the mainland it is relatively little impacted upon by offshore fishery and human activity. In our study the marine sponge *H. perleve* inhabiting such environments was sampled to investigate the associated *Actinobacteria*.

Previous studies have been mainly focused on marine sponges distributed along the coast of the South China Sea (Jiang et al. 2007; Xin et al. 2008). The marine sponges inhabiting the open sea are rarely investigated. The present study allows us to compare the actinobacterial community of marine sponges from offshore and open sea in the South China Sea. A previous study investigated an offshore sponge in

the South China Sea and detected four phylogenetic divisions (*Mycobacterium*, *Acidimicrobium*, *Cellulosimicrobium* and unidentified *Actinobacteria*) from the clone library (Xin et al. 2008). The dominant group was *Mycobacterium*, representing 70% of the clones. This is consistent with our finding that *Mycobacterium* predominated as 72% of all clones. This suggests that *Mycobacterium* may be the dominant *Actinobacteria* in the South China Sea sponges. In contrast, the open sea sponge symbionts showed higher actinobacterial diversity than were recovered from the offshore sponge. Another marked difference is reflected in the cultured actinomycetes. In our earlier study on culturable *Actinobacteria* within the marine sponge *Haliclona* sp. it was found that the majority of the strains belonged to *Streptomyces* and many strains have terrestrial actinobacterial characteristics (Jiang et al. 2007). Therefore, they probably originate from terrestrial run-off. A different result was observed in this study, where most of the cultured strains belonged to *Salinispora* that was indigenous to the tropical and subtropical marine environment. The open sea sponges in the South China Sea are likely better hosts for true marine *Actinobacteria* and can provide more opportunities for natural product search and discovery.

In conclusion, a broad diversity of *Actinobacteria* associated with the marine sponge *H. perleve* from the South China Sea was described by using both culture-independent and culture-dependent methods. At least 14 actinobacterial genera were present in *H. perleve*. Moreover, *Salinispora* was isolated and cultivated from a South China Sea sponge for the first time. The cultured *Salinispora* strains are a valuable resource, which can be used in future research on metabolites and biosynthetic pathways. This study lays the ground work for further understanding the actinobacterial biodiversity and biogeography associated with marine sponges.

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