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ORIGINAL PAPER

Culturable actinobacteria from the marine sponge *Hymeniacidon perleve*: isolation and phylogenetic diversity by 16S rRNA gene-RFLP analysis

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Abstract A total of 106 actinobacteria associated with the marine sponge *Hymeniacidon perleve* collected from the Yellow Sea, China were isolated using eight different media. The number of species and genera of actinobacteria recovered from the different media varied significantly, underlining the importance of optimizing the isolation conditions. The phylogenetic diversity of the actinobacteria isolates was assessed using 16S rRNA gene amplification–restriction fragment length polymorphism (RFLP) analysis of the 106 strains with different morphologies. The RFLP fingerprinting of selected strains by *Hha*I-digestion of the 16S rRNA genes resulted in 11 different patterns. The *Hha*I-RFLP analysis gave good resolution for the identification of the actinobacteria isolates at the genus level. A phylogenetic analysis using 16S rRNA gene sequences revealed that the isolates belonged to seven genera of culturable actinobacteria including *Actinoalloteichus, Micromonospora, Nocardia, Nocardiopsis, Pseudonocardia, Rhodococcus*, and *Streptomyces*. The dominant genus was *Streptomyces*, which represented 74% of the isolates. Three of the strains identified are candidates for new species.

Keywords Marine sponge · Actinobacteria · RFLP · *Streptomycetes* · *Nocardiopsis* · *Pseudonocardia* · *Nocardia* · *Micromonospora* · *Rhodococcus* · *Actinoalloteichus*

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Introduction

The class Actinobacteria is a very important group of bacteria that has considerable value as prolific producers of antibiotics and other therapeutic compounds. Actinobacteria produce over half of the bioactive compounds in the Antibiotic Literature Database (Lazzarini et al. 2000). However, with the intense exploitation of terrestrial actinobacteria over many years, the discovery rate of novel bioactive compounds has fallen steadily, with an estimated 95% rediscovery rate of known compounds (Fenical et al.1999). To solve this problem, the isolation of novel actinobacteria from marine environments has

been a fruitful area of research in the past decade (Stach and Bull 2005). However, little is known about the diversity of actinobacteria from marine samples compared to the diverse range of actinobacteria isolated from terrestrial environments. Increasing numbers of both culture-based studies and culture-independent molecular studies show that many actinobacteria exist in marine environments such as sediments, seawater, and marine invertebrates (Cifuentes et al. 2000; Hentschel et al. 2001; Mincer et al. 2002; Piza et al. 2004; Webster et al. 2004). A major novel marine actinobacterial taxon in ocean sediments was found to have widespread, persistent populations in ocean systems (Mincer et al. 2002) and some taxa have no counterparts in terrestrial environments (Hentschel et al. 2001; Montalvo et al. 2005; Webster et al. 2001). It is becoming evident that marine habitats are an abundant, novel source of actinobacteria for discovering natural products.

Of the marine inhabitants investigated, the lowest marine metazoan invertebrates, sponges in the phylum *Porifera*, are of great interest for discovering novel actinobacteria. As sessile filter-feeding animals, sponges are the largest sources of marine bioactive metabolites, accounting for up to 40% of all known natural marine products (Lee et al. 2001). The isolated metabolites have antimicrobial, antitumoral, antiviral, and enzyme-inhibitory activities (Blunt et al. 2003, 2004; Faulkner 2002). It was hypothesized that the high occurrence and diversity of bioactive compounds could be attributed partially, if not largely, to the abundant bacteria associated with marine sponges (Webster et al. 2001). Some studies have proposed that sponge-associated bacteria are the true origin of some sponge-derived natural products (Jayatilake et al. 1996; Oclarit et al. 1994), although the demonstration of this is very difficult. The sponges *Aplysina cavernicola* and *Ceratoporella nicholsoni* harbor large numbers of bacteria that amount to 38 and 57% of their biomass volume, respectively (Friedrich et al. 1999; Willenz et al. 1989). The number of bacteria in sponges is estimated to exceed that in seawater by two to four orders of magnitude (Friedrich et al. 1999). However, few studies have investigated the diversity, distribution and ecology of actinobacteria from marine sponges, although there are several reports that they are abundant (Hentschel et al. 2002; Imhoff and Stöhr 2003; Webster et al. 2001). Recent studies using both culture-independent molecular approaches and culture-based methods demonstrated that novel, abundant actinobacteria assemblages are associated with the sponges *Rhopaloeides odorabile* (Webster et al. 2001) and *Halichondria panacea* (Imhoff and Stöhr 2003). However, the investigation of sponge-associated actinobacteria is presently limited to a few sponge species out of the over 15,000 marine species, which is insufficient to provide a general understanding of their diversity, distribution, and ecology for further exploitation of this novel source of actinobacteria. Furthermore, it is very difficult to verify if the actinobacteria are specifically associated with the host marine sponges.

To better understand the actinobacteria diversity associated with marine sponges, we attempted to isolate and cultivate actinobacteria from an inter-tidal marine sponge *Hymeniacidon perleve* for further physiological studies and to discover bioactive natural products. *H. perleve* is widely distributed along the coast of the Yellow Sea near Dalian, China. We recently isolated many antibacterial and antitumor compounds from this sponge (Xue et al. 2004). A culture-independent molecular approach has indicated a high diversity of the associated bacteria, although few actinobacteria were identified (Xu et al. 2004). We wanted to discover whether the sponge harbors actinobacteria that were not easily detected because of a possible low abundance of Gram-positive actinobacteria. In this study, we isolated and cultured actinobacteria from *H. perleve* using a variety of media and their phylogenetic diversity was assessed using 16S rRNA gene sequencing and RFLP analysis.

Materials and methods

Sponge collection

Specimens of the marine sponge *H. perleve* were collected manually in March 2003 from the inter-tidal beach of the Yellow Sea at Dalian, China (38°52′ N, 121°41′ E). Sponge specimens were placed in plastic bags containing seawater and immediately transported to the laboratory.

Media and isolation of actinobacteria

To maximize the isolation of actinobacteria, 12 different types of media for the isolation of actinobacteria were selected from the literature (Table 1). All media were supplemented with a final concentration of 50 μg ml^{-1} potassium dichromate ($K_2Cr_2O_7$) and 15 μg ml^{-1} nalidixic acid to facilitate the isolation of slow-growing actinobacteria. $K_2Cr_2O_7$ inhibits fungal growth (Yang et al. 1995), while nalidixic acid inhibits many fast-growing Gram-negative bacteria (Webster et al. 2001). All media contained Difco Bacto agar (18 g l^{-1}) at pH 7.0. To promote the growth of selected sponge-associated actinobacteria, all media were supplemented with 1% sponge water extract prepared by grinding 20 g of sponge tissue in a mortar containing 20 mL of sterile seawater; the homogenate was filtered through gauze, sterilized for 15 min at 121°C and centrifuged to collect the supernatant as sponge water extract.

To isolate sponge-associated actinobacteria, the freshly collected sponge specimens were rinsed five times in sterile seawater to remove transient and loosely attached bacteria. The washed specimens were then cut into pieces measuring ca. 1 cm^3 and thoroughly homogenized in a sterile mortar with 10 volumes of sterile seawater. A 10-fold dilution series was made and plated in triplicate on agar plates. The inoculated plates were incubated at 28°C for 2–4 weeks.

Strain culture, DNA isolation and 16S rRNA gene amplification

All actinobacterial isolates were cultured on Gauze's No. 1 agar (Labeda et al. 1990). Slow-growing strains were cultured on TSA agar (Difco, USA). The strains were selected by the morphological differences based on visible examination of the growth characteristics, aerial mycelium, substrate mycelium and diffusible pigments. For DNA extraction, the selected strains were cultured in trypticase soy broth (TSB) for 4 days, and total genomic DNA was extracted from each strain using a previously described method (Lee et al. 2003). From the genomic DNA, nearly full-length 16S rRNA gene sequences were amplified by polymerase chain reaction (PCR) using primers F8 and R1492 (Table 2). The PCR mixture consisted of 5 μl of 10× buffer (Mg^{2+} free), 5 μl of 2.5 μM $MgCl_2$,

Table 1 Composition of the 12 different media for isolation of actinobacteria from the marine sponge *H. perleve*

Medium	Formula[a]	Reference	Number of isolates
M1	10 g soluble starch, 4 g yeast extract, 2 g peptone, 18 g agar, and 1 l of natural seawater	Mincer et al. (2002)	15
M2	6 mL 100% glycerol, 1 g arginine, 1 g K_2HPO_4, 0.5 g $MgSO_4$, 18 g agar, and 1 l of natural seawater	Mincer et al. (2002)	26
M3	4 g yeast extract, 10 g malt extract, 4 g glucose, 18 g agar, and 1 l of natural seawater	Webster et al. (2001)	3
M4	0.1 g L-asparagine, 0.5 g K_2HPO_4, 0.001 g $FeSO_4$, 0.1 g $MgSO_4$, 2 g peptone, 4 g sodium propionate, 20 g NaCl, 18 g agar, and 1 l of water	This study	20
M5	10 g beef extract, 4 g peptone, 10 g brain heart infusion, 5 g yeast extract, 5 g glucose, 15 g K_3PO_4, 20 g NaCl, 1 g soluble starch, 1 g $(NH_4)_2SO_4$, 1 g cysteine, 0.2 g $MgSO_4$, 0.01 g $CaCl_2$, 18 g agar, and 1 l of water	This study	6
M6	4 g beef extract, 4 g peptone, 1 g yeast extract, 10 g glucose, 20 g NaCl, 18 g agar, and 1 l of water	Webster et al. (2001)	1
M7	2 g peptone, 0.1 g asparagine, 4 g sodium propionate, 0.5 g K_2HPO_4, 0.1 g $MgSO_4$, 0.001 g $FeSO_4$, 5 g glycerol, 20 g NaCl, 18 g agar, and 1 l of water	Webster et al. (2001)	19
M8	4 g yeast extract, 15 g soluble starch, 1 g K_2HPO_4, 0.5 g $MgSO_4$, 20 g NaCl, 18 g agar, and 1 l of water	Webster et al. (2001)	16
M9	6 g glucose, 2 g chitin, 18 g agar, and 1 l of natural seawater	Mincer et al. (2002)	0
M10	2 g chitin, 18 g agar, and 1 l of natural seawater	Mincer et al. (2002)	0
M11	18 g agar and 1 l of natural seawater	Mincer et al.(2002)	0
M12	20 g soluble starch, 1 g KNO_3, 0.5 g K_2HPO_4, 0.5 g $MgSO_4$, 0.01 g $FeSO_4$, 20 g NaCl, 18 g agar, and 1 l of water	Labeda and Shearer (1990)	0

[a] All the media except those prepared in seawater were adjusted to a final pH 7.0

Table 2 Oligonucleotide primers used in this study[a]

Primer[b]	Sequence (5′–3′)	Source of reference
F8	GAGAGTTTGATCCTGGCTCAG	Webster and Hill (2001)
R1492	CGGCTACCTTGTTACGAC	Webster and Hill (2001)
F337	GACTCCTACGGGAGGCAGCAG	This study
F785	GGATTAGATACCCTGGTA	This study
F1225	ACACACGTGCTACAATGG	This study
R518	GTATTACCGCGGCTGCTGG	This study

[a]Primers F8 and R1492 were used for the PCR amplification of 16S rRNA gene of actinobacteria strains; Primers F337, F785, F1225 and R518 were used for the sequencing analysis using ABI PRISM 3100 automated sequencer

[b]All primers are named after their respective 16S rDNA gene priming sites (*Escherichia coli* numbering)

8 μl of dNTP mixture (2.5 μM each), 1 μl of each primer, 1 μl of template DNA and 0.5 μl of LA *Taq* DNA polymerase (5 U/ μl) (TaKaRa, China) in a final volume of 50 μl. PCR was performed in a thermal cycler (Biometra, Germany) using an initial denaturation at 95°C for 5 min, followed by 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min and a final extension at 72°C for 7 min (Lee et al. 2003).

Sequencing and phylogenetic analysis

The amplified PCR products were purified using a High Pure PCR Product Purification Kit (Roche, IN). Nearly full-length sequences of the amplified 16S rRNA genes (>1450 bp) were obtained using an ABI PRISM 3100 automated sequencer (PE Applied Biosystems, CA). The sequences were edited using PHYDIT (Chun 1995), and a blast search of the National Center for Biotechnology Information (NCBI) was performed to identify the nearest neighbor to the amplified sequence. The sequences were aligned with actinobacteria 16S rRNA gene data retrieved from the NCBI website to create a matrix using CLUSTALW (Thompson et al. 1994). The tree topologies were evaluated by bootstrap analyses based on 1000 replications with PHYLIP (Felsenstein 1993) and phylogenetic trees were inferred using the neighbor-joining method (Saitou and Nei 1987).

RFLP analysis

Each amplified 16S rRNA gene was digested using the four-cutter restriction enzyme *Hha*I (Promega, WI) for 2 h. After electrophoresis on 2% agarose gels at 50 V for 3 h, band patterns were captured and analyzed using a BioDocAnalyze system (Biometra, Germany). The RFLP patterns based on the 16S rRNA gene sequences were also analyzed using Vector NTI 8 (Informax, Germany) and compared with the electrophoresis data.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences determined for the 16 representative strains in this study were deposited in the GenBank database with the accession numbers indicated in parentheses: HPA1 (DQ144212), HPA124 (DQ144213), HPA135 (DQ144216), HPA136 (DQ144217), HPA140 (DQ144218), HPA160 (DQ144220), HPA173 (DQ144221), HPA177 (DQ144222), HPA180 (DQ144223), HPA19 (DQ144224), HPA192 (DQ144225), HPA53 (DQ144228), HPA66 (DQ144230), HPA72 (DQ144231), HPB43 (DQ144233), and HPB6 (DQ144234).

Results

Effect of isolation media on the recoverability of marine sponge-associated actinobacteria

Among 12 different actinobacteria isolation media tested, actinobacteria were recovered on 8 media only, producing a total of 106 strains with different morphologies from *H. perleve*. Significant differences in the total number of isolates recovered were observed among the 8 different media (Fig. 1). M2 produced the highest recovery with 26 isolates, followed by M4 (20 isolates), M7 (19 isolates), M8 (16 isolates), M1 (15 isolates), M5 (6 isolates), M3 (3 isolates), and M6 (1 isolate). All these media have

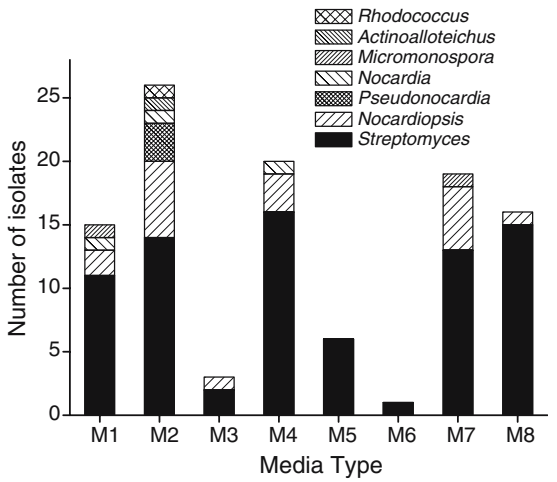

Fig. 1 The distribution of genera and the diversity of actinobacteria recovered on the eight isolation media

been widely used to isolate actinobacteria from diverse terrestrial environments (Labeda and Shearer 1990; Zakharova et al. 2003).

One hundred and six putative actinobacterial strains were selected from the isolation plates and sub-cultured for further analysis. The 16S rRNA genes of the 106 strains were sequenced and the genera of specific strains were determined after Blast analysis. It was found that the actinobacteria diversity recovered in the different media also varied considerably (Fig. 1). M2 had the best recoverability, with six of the total of seven actinobacteria genera recovered. M1 recovered four genera; M4 and M7 recovered three genera; M3 and M8 recovered two genera; and M5 and M6 recovered only one isolate of the genus of *Streptomyces*. *Streptomyces* were recovered on all eight media that yielded growth, making it the most abundant culturable actinobacteria group found in *H. perleve*. *Nocardiopsis* was recovered from six media, and was the second dominant genus (Fig. 1). The genus *Micromonospora* was only isolated in two media: M1 and M7. Moreover, M4 and M7, which had the high number of isolates, recovered only three genera.

Phylogenetic analysis based on 16S rRNA gene sequences

Of the isolated 106 actinobacteria, 74% (78/106) belonged to the genus *Streptomyces*, which was the dominant actinobacteria genus in *H. perleve*, and 26% (28/106) belonged to six other actinobacterial genera: *Nocardiopsis* (18 strains), *Pseudonocardia* (3 strains), *Nocardia* (3 strains), *Micromonospora* (2 strains), *Rhodococcus* (1 strain), and *Actinoalloteichus* (1 strain). The 16S rRNA genes of the 106 strains were sequenced nearly in full. Seven taxa represented by a single strain and 9 taxa containing multiple strains of identical 16S rRNA gene sequence were identified (Table 3). Cumulatively, a total of 16 different taxa were thus finally identified and their 16S rRNA gene sequences were used to construct phylogenetic trees (Figs. 2 and 3). The phylogenetic analyses indicated that the *Streptomyces* spp. isolated from *H. perleve* were diversely distributed within this genus (Fig. 2). Strain HPA124 showed the closest homology with *Streptomyces gibsonii* (97.95%), while HPB43 was closest to *Streptomyces somaliensis* (97.41%). Although the similarities were more than 97%, the two strains may still be new species, due to the complexity of this genus and the present lack of a definitive taxonomic system. The phylogenetic relationships of the 16S rRNA gene sequences from the less frequently recovered actinobacterial genera were also examined (Fig. 3). The greatest similarity of the HPA177 16S rRNA gene sequence was 96.72% with that of the known strain *Actinoalloteichus cyanogriseus* and 97.14% with that of *Actinoalloteichus spitiensis* and it is thus likely that this strain will prove to represent the third species in the genus *Actinoalloteichus*.

*Hha*I-based 16S rRNA gene-RFLP analysis

The PCR products of 16S rRNA genes from actinobacteria isolated from *H. perleve* were each about 1.6 kb long. After purification, the amplicons were digested with the four-cutter endonuclease *Hha*I for RFLP analysis on agarose electrophoresis. For the 106 strains analyzed, 11 different RFLP patterns were differentiated (Table 3; Fig. 4). The main digestion products were 250–550 bp in size. When correlated with the 16S rRNA gene sequence analysis of the 106 strains, it was found that each typical RFLP pattern represented a group of isolated actinobacteria, as shown in Table 3. Strains yielding RFLP patterns 1, 2, 3, and 4 belonged to genus *Streptomyces*; Strains yielding patterns 5 and 6 belonged to *Nocardiopsis*; The strain yielding pattern 7 belonged to *Pseudonocardia*; The strain yielding

Table 3 Restriction fragment length polymorphism (RFLP) fingerprinting patterns and sequence relationships of 16S rRNA gene from actinobacteria isolated from the marine sponge *H. perleve*

RFLP Pattern	Representative strain		The most similar species	Similarity (%)	Number of strains
1	I	HPA53	*Streptomyces tendae*	98.86	21
	II	HPA136	*Streptomyces aureofaciens*	99.79	3
	III	HPA72	*Streptomyces gougerotii*	99.93	1
	IV	HPA135	*Streptomyces argenteolus*	99.93	1
	V	HPA124	*Streptomyces gibsonii*	97.95	1
	VI	HPB43	*Streptomyces rimosus*	97.41	1
2	I	HPA1	*Streptomyces coelicolor*	99.28	42
3	I	HPA66	*Streptomyces caviscabies*	99.50	7
4	I	HPA160	*Streptomyces paradoxus*	99.07	1
5	I	HPA173	*Nocardiopsis lucentensis*	98.7	7
6	I	HPA19	*Nocardiopsis dassonvillei*	99.43	11
7	I	HPA192	*Pseudonocardia antarctica*	99.85	3
8	I	HPA180	*Nocardia salmonicida*	99.78	3
9	I	HPB6	*Micromonospora aurantiaca*	100	2
10	I	HPA177	*Actinoalloteichus cyanogriseus*	96.72	1
11	I	HPA140	*Rhodococcus opacus*	98.47	1
Total					106

pattern 8 belonged to *Nocardia*; The strain yielding pattern 9 belonged to *Micromonospora*; The strain yielding pattern 10 belonged to *Actinoalloteichus*; and the strain yielding pattern 11 belonged to *Rhodococcus*. Therefore, using the *Hha*I-based 16S rRNA gene-RFLP fingerprinting analysis, the actinobacteria isolated from the marine sponge *H. perleve* could be identified to the genus level. Representatives of two genera (*Streptomyces* and *Nocardiopsis*) could be identified to the subgenus level, as they were grouped into four and two patterns, respectively. In addition, RFLP fingerprinting was simulated using the Software Vector NTI. By comparing with the experimental results, the software analysis demonstrated high reliability of the RFLP fingerprints (data not shown).

Discussion

We isolated and cultured 106 representatives of seven genera of Actinobacteria from the marine sponge *H. perleve*. This is the first report to recover such a high diversity of culturable actinobacteria from any marine sponges. In addition, this is also the first report of the isolation and cultivation of strains of the genera *Actinoalloteichus*, *Micromonospora*, *Nocardiopsis*, *Nocardia* and *Rhodococcus* from marine sponges. Previous studies have isolated strains of *Brachybacterium*, *Gordonia*, *Micrococcus* and *Streptomyces* from the sponges *Xestospongia muta*, *R. odorabile* and other unidentified sponges (Bultel et al. 1998; Lee et al. 1998; Montalvo et al. 2005). Of the strains identified from *H. perleve*, three may represent new species. Using the polyphasic taxonomic analyses, the strain HPA177 has already been identified as a candidate for new species of *Actinoalloteichus* (Zhang et al. 2005, maunscript submitted). Together with the previous demonstration of a high abundance of actinobacteria using culture-independent molecular analysis (Hentschel et al. 2002; Imhoff and Stöhr 2003; Webster et al.2001), the presence of a high diversity of culturable actinobacteria within *H. perleve* suggests that sponges have potential as an excellent source of actinobacteria.

The isolation media were highly selective for the recovery of actinobacteria. Of the 12 actinobacteria isolation media tested, the total number and diversity of isolates recovered differed markedly, with six genera recovered on M2, but none on four of the media (Fig. 1). The common feature of the 4 media that yielded no actinobacteria is that they do not contain free amino acids, when compared with the other 8 media. M2 is a widely used isolation media, which has been successfully applied in the terrestrial environment. The main carbon and nitrogen sources in M2 are glycerol and arginine, which are preferred

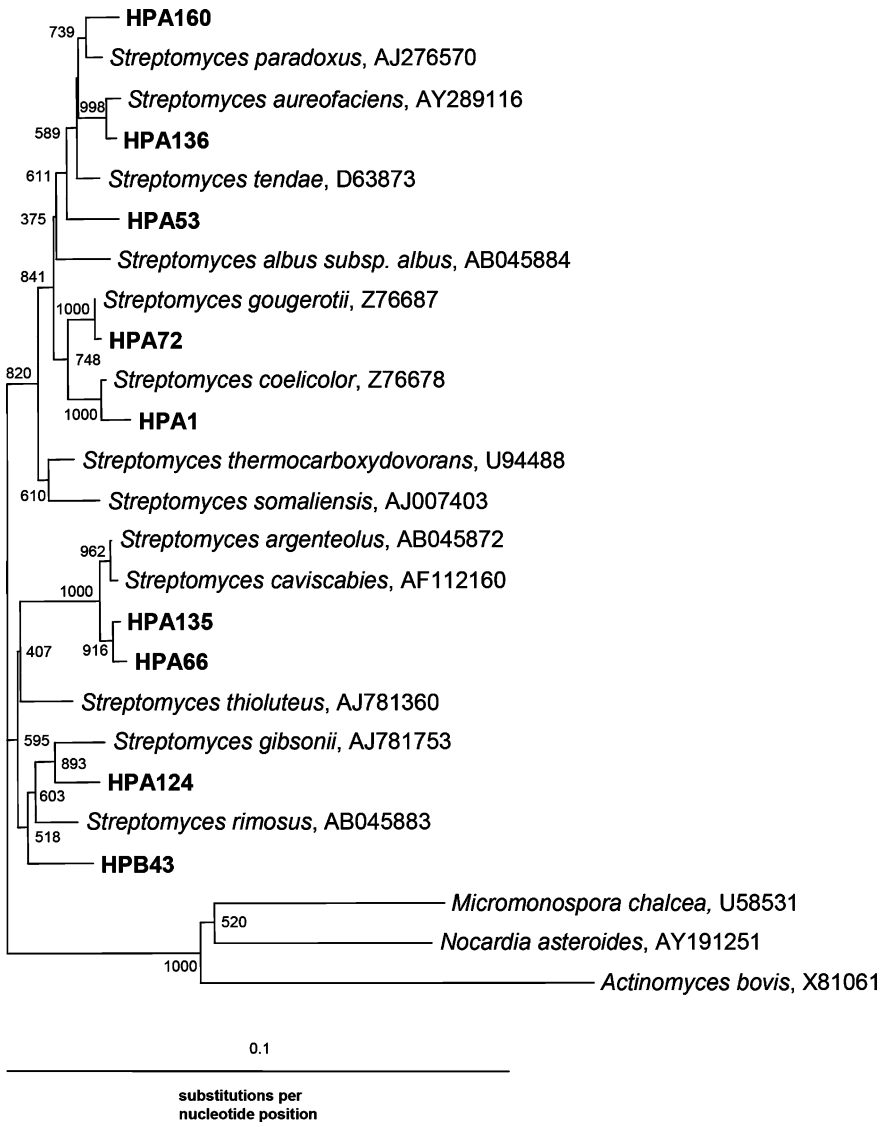

Fig. 2 Phylogenetic relationships determined from the 16S rRNA gene sequences of *Streptomyces* isolated from the marine sponge *H. perleve*. Bootstrap values calculated from 1000 resamplings using neighbor-joining are shown at the respective nodes when the calculated values were 50% or greater. *Micromonospora chalcea* (U58531), *Nocardia asteroides* (AY191251), and *Actinomyces bovis* (X81061) were used as outgroups

nutrients for actinobacteria (Labeda and Shearer 1990). As far as the marine environment is concerned, marine prokaryotes grew better in the inorganic media than in complex organic media (Macleod 1965). This preference was also observed for the cultivation of the ubiquitous marine bacterioplankton clade SAR11 (Rappé et al. 2002). Our data would further support this notion that actinobacteria from marine sponge are recovered better on predominantly inorganic isolation media. Similarly, it was reported that different media have diverse effects on actinobacteria isolation from the sponge *R. odorabile*, with two inorganic rich media showing the best recoverability (Webster et al. 2001). In contrast, the proportion of actinobacteria increased significantly from 20 to 40% at lower substrate concentrations when isolating bacteria from *H. panacea* (Imhoff and Stöhr 2003). These conflicting results imply a complex relationship between the isolation medium and the recoverability of culturable actinobacteria from marine sponges. Given that it is estimated that less than 1% of bacteria can be cultured at present (Webster et al. 2001), the formulation of appropriate isolation media and culture conditions is critical for improving the recovery of actinobacteria. To understand the diversity and distribution of the culturable actinobacteria community within marine sponges, a well-designed isolation protocol with

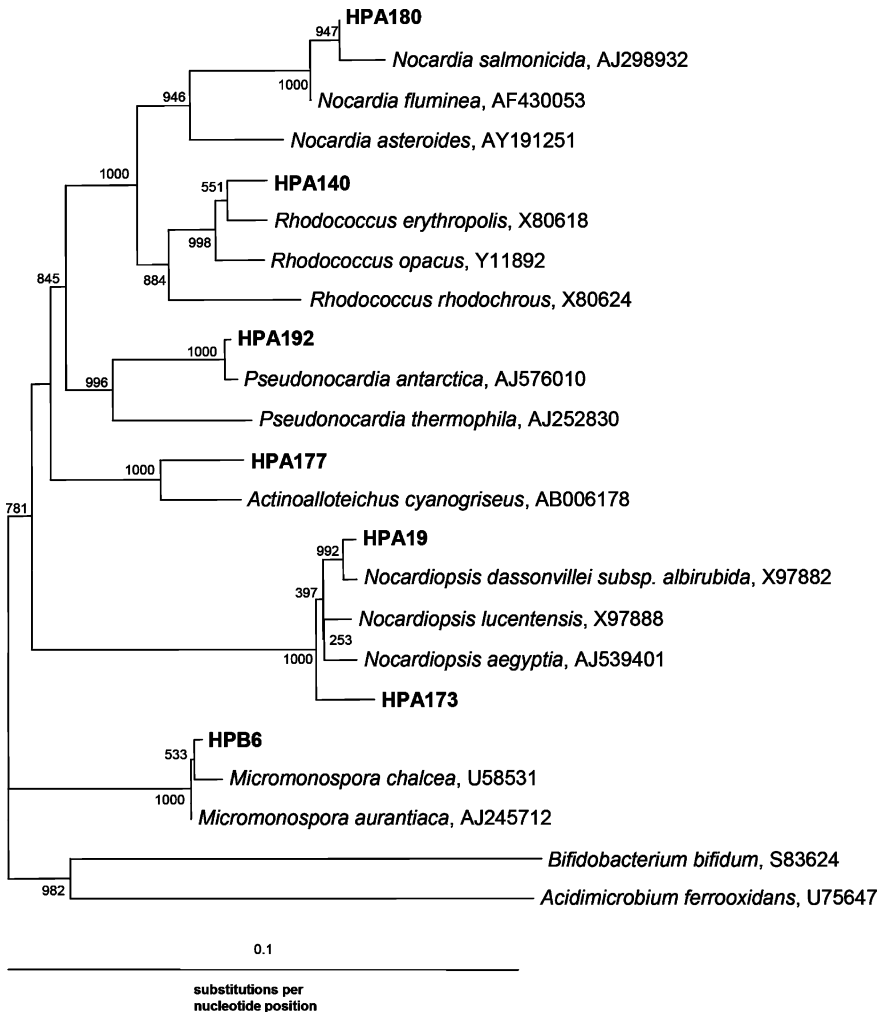

Fig. 3 Phylogenetic relationships determined from the 16S rRNA gene sequences of *Nocardiopsis*, *Pseudonocardia*, *Nocardia*, *Micromonospora*, *Rhodococcus*, and *Actinoalloteichus* isolated from the marine sponge *H. perleve*. Bootstrap values calculated from 1,000 resamplings using neighbor-joining are shown at the respective nodes when the calculated values were 50% or greater. *Bifidobacterium bifidum* (S83624) and *Acidimicrobium ferrooxidans* (U75647) were used as outgroups

multiple isolation media and culture conditions is essential.

RFLP analysis of the 16S rRNA gene is a rapid and inexpensive method that can be applied to studies of the identity and diversity of microbial communities. Actinobacterial strains could be identified at the genus level using four restriction endonucleases without sequencing (Cook and Meyers 2003). When there are too many isolates and the sequencing costs of all strains are high, RFLP analysis can effectively reduce the number of isolates needing to be sequenced. In the current study, we only used one restriction endonuclease *Hha*I that specifically recognizes and cuts the sequence ''GCGC''. The resulting 11 different RFLP patterns obtained allowed us to effectively differentiate the strains into distinct groups of actinobacteria. When the *Hha*I RFLP fingerprints were compared with the corresponding 16S rRNA gene sequences, the isolated actinobacteria could be identified at the genus level and for two genera, *Streptomyces* and *Nocardiopsis*, at the subgenus level (Table 3). This rapid and convenient method can be very useful in the large scale identification of actinobacteria isolates. However our results also indicated that even within one single RFLP pattern, some diversity was apparent from the 16S rRNA sequencing (Table 3). Caution must be therefore taken when using the RFLP approach for a complete phylogenetic analysis.

Streptomyces was found to be the dominant genus of culturable actinobacteria from *H. perleve*, with 78 sequenced strains (73.6% of all strains). *Streptomyces* was also the easiest genus to isolate and culture, as it was recovered from eight media (Fig. 1). The genus

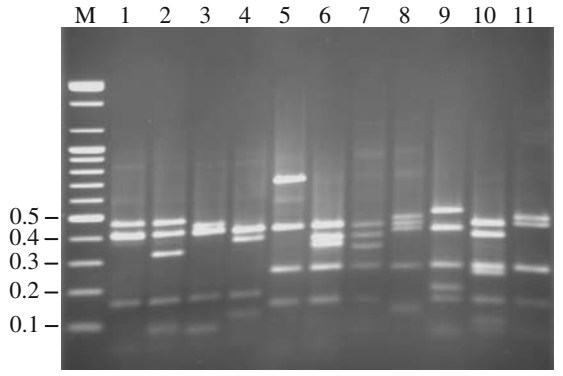

Fig. 4 The restriction fragment length polymorphism (RFLP) patterns of the 16S rRNA gene PCR products of actinobacteria isolated from the marine sponge *H. perleve* digested with the restriction enzyme *Hha*I. M is the molecular marker and the number represents the units of marker, kb. The lane number corresponds to the RFLP pattern listed in Table 3

Streptomyces is widely distributed in nature and is a source of commercial enzymes and therapeutically useful bioactive molecules (Stach and Bull 2005). Increasingly, *Streptomyces* strains have been isolated from marine environments (Colquhoun et al. 1998) and many novel bioactive compounds with several unique structures have been isolated (Itoh et al. 2003; Sanchez et al. 2003). Some bioactive compounds have also been isolated from marine sponge-associated *Streptomyces* (Lee et al. 1998). In our laboratory, the potential bioactive compounds maculosin (antibacterial) and chromomycin A3 (antitumoral) have been isolated from the *Streptomyces* strains HPB37 and HPA109 associated with *H. perleve*, respectively (data not shown). The *Streptomyces* strains isolated from *H. perleve* had four different RFLP patterns, reflecting the high diversity of this genus. Many different *Streptomyces* species have very similar 16S rRNA gene sequences. It was recently reported that the genus could be divided into more than 50 clusters using 16S-ITS RFLP fingerprinting analysis (Lanoot et al. 2005). Therefore, this genus may eventually be divided into several genera.

It has been reported that the ''*Micromonospora–Rhodococcus–Streptomyces*'' group is the dominant, ubiquitous actinobacteria in marine environments (Maldonado et al. 2005). Representatives of all three of these taxa were isolated from the marine sponge *H. perleve*, indicating a general feature of marine inhabitants. However, *H. perleve* yielded four other less frequently recovered actinobacteria, indicating that marine sponges are potentially unique sources of novel actinobacteria. The 16S rRNA gene sequences of eighteen isolates showed greatest similarity to those of members of the genus *Nocardiopsis* and consisted of two RFLP patterns that represented two *Nocardiopsis* groups. Many new species of this widely distributed genus have recently been isolated from the marine environment (Peltola et al. 2001; Sabry et al. 2004). Strain HPA177 had similarity of 97.14% with *Actinoalloteichus spitiensis* (Singla et al. 2005), and 96.72% with *Actinoalloteichus cyanogriseus* (Tamura et al. 2000), the only two published strains in this genus. The genus has not been reported from the sea and HPA177 is the first *Actinoalloteichus* strain isolated from the marine environment. Many of these isolated strains have terrestrial actinobacterial characteristics: for example, HPA177 did not have a strict requirement of high concentrations of NaCl and grew well in normal actinobacterial cultural media (Zhang et al. 2005, manuscript submitted). Given that marine sponge *H. perleve* is an inter-tidal sponge species, the diversity of actinobacteria from *H. perleve* may be related to its inter-tidal living environment and filter-feeding behavior. Several published studies on actinobacteria from the inter-tidal marine environment also discussed the origin of some ''obligate marine actinobacteria'' (Jensen et al. 1991; Hobel et al. 2005).

In our earlier studies of bacterial diversity in *H. perleve* using a culture-independent molecular approach, the predominant bacterial groups identified were α-Proteobacteria and γ-Proteobacteria, which constituted over 62% of the 21 sequenced clones (Xu et al. 2004), with only one clone belonging to the actinobacteria. The sequence analysis of this clone indicated that it was most closely related to the genus *Sporichthya*. However, our current study yielded representatives of seven actinobacterial genera but not of the genus *Sporichthya*. This observed difference has been noted previously in comparisons between the genetically verified diversity and the culturable diversity of bacteria from sponges (Imhoff and Stöhr 2003; Webster et al. 2001). It was reported that the most abundant bacteria isolated from the Great Barrier Reef sponge *R. odorabile* were related to α-Proteobacteria (Webster and Hill 2001); however, a molecular genetic analysis of bacterial diversity indicated that actinobacteria were the

dominant group (Webster et al. 2001). Similar results were also reported concerning the isolation of actinobacteria from the marine sponges *Xestospongia muta* and *X. testudinaria*, in that the strains isolated were not represented in the clone libraries (Montalvo et al. 2005). Directed by the information from the culture-independent methods, actinobacteria could also be cultured using suitable isolation approaches (Mincer et al. 2005; Rappé et al. 2002). It is necessary to carry out detailed molecular analysis, e.g. using actinobacteria-specific primers, to reveal the true diversity even if actinobacteria are present as minor components of the total community. Fluorescent *in situ* hybridization analysis (FISH) with a probe specific for actinobacteria (Friedrich et al. 1999) could be applied in future studies to investigate the numbers and location of actinobacteria associated with *H. perleve*. Therefore, it is important to recognize the advantages and limitations of both cultivation-based and genetic approaches in revealing the actinobacterial diversity within marine sponges.

In conclusion, the marine sponge *H. perleve* was found to harbor the highest diversity of culturable actinobacteria reported so far, with representatives of seven genera present and *Streptomyces* the dominant genus. The recovery of culturable actinobacteria depends largely on using appropriate isolation media and culture conditions. The isolation of culturable actinobacteria from marine sponges can contribute to our knowledge of sponge-associated actinobacteria and further increase the pool of actinobacteria available for bioactive natural products screening.

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