Phylogenetic Diversity of Bacterial Community Associated with the Marine Sponge *Halichondira nigrocutis* Collected off Southwest Indian Coast

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ABSTRACT

This study aimed to evaluate the bacterial communities associated with the marine sponge *Halichondria nigrocutis* present in Indian waters by both cultivation and cultivation-independent techniques. Results using cultivation method showed that bacteria belonging to *Bacillus*, *Acinetobacter* and *Vibrio* spp. to be the predominant groups. Metagenomic study of sponge-associated bacteria by cultivation independent approach, involving cloning and sequencing of the 16S rDNA gene generated sequences that were subjected to phylogenetic analysis. Results demonstrated the community structure to be represented by the phyla *Proteobacteria* (*alpha-, gamma- and delta-*classes), *Cyanobacteria, Actinobacteria* and *Firmicutes,* with isolates belonging to *alpha-proteobacterial* group to be predominating. To our knowledge this study appears to be the first to record cultivable and uncultivable bacterial groups associated with *H.nigrocutis* from Indian waters.

Key words: *Halichondria nigrocutis*, Marine sponge, Phylogenetic diversity, Sponge-associated bacteria

Sponges classified in the phylum Porifera are primitive metazoans that inhabit marine and freshwater habitats. Of the estimated 15,000 sponge species described so far (Hooper and van Soest, 2002), >486 sponges are recorded from the Indian waters (Thomas, 1998). Sponges are sessile filter feeders and possess efficient capacity to filter out large volumes of water per day through their aquiferous channel system, as a result of which they capture vast amount of planktons and microbes that form the key components of their natural diet (Hentschel *et al*. 2003; Webster and Taylor, 2012). It is well documented that some of the microbes can associate themselves as 'symbionts' capable of growing in the microenvironment of the sponge mesohyl reaching a density of 10^5 -10^9 depending on the sponge type (Hentschel *et al*. 2003; 2006; Imhoff and Stöhr 2003; Simister and Deines 2012; Webster and Taylor 2012). Studies show the bacteria associated with sponge to be host specific, although their role in the life history of sponge is unclear. In recent years, the study of sponge-bacterial symbiosis has gained considerable interest as the bioactive secondary metabolites produced by sponges are thought to be a result of compounds biosynthesized by specific sponge associated microbial groups rather than being of sponge origin. Many of the bioactive molecules have proven to be pharmaceutically important as anticancer, antiviral and anti-inflammatory molecules (Laport *et al*. 2009; Sagar and Minneman, 2010; Kim and Dewapriya 2012). While several of these compounds have been successfully approved for use in treating diseases, there are many in pipeline that are undergoing preclinical/clinical trials for various diseases (Laport *et al*. 2009; Hill and Fenical 2010; Sagar *et al*. 2010).

In India, the sponge fauna is dominated by the species belonging to the class *Demospongiae* (Venkataraman and Wafer 2005). Although the bacterial communities associated with Demosponge species have been recorded from several parts of the world (Taylor *et al*. 2007), there are very few such studies published for species in Indian waters. Further, the sponges belonging to the class Demospongiae are known to produce a large number of secondary metabolites that are being used as natural therapeutics (Faulkner 1998). Hence, the purpose of this study was to document the bacterial community and diversity in the marine sponge *Halichondira nigrocutis* (class *Demospongiae*, order *Halichondrida*, family *Halichondriidae*) found in the intertidal zone of West Coast of India.

MATERIALS AND METHODS

***Sample collection*:** Specimens of the marine sponge *H. nigrocutis* collected during the lowest low tide from the intertidal zone of Majaahalli beach, Karwar, Karnataka, India (latitude 14° 56' 23.74" N and longitude 74° 04'

30.77" E) were cut into individual pieces, transferred to a sterile plastic bag containing seawater, and transported to the laboratory on ice for further processing. Each sponge sample was rinsed with filtered and autoclaved seawater until they were visibly free of debris. The sponge surface was then disinfected with a rapid wash with 70% ethanol followed by immediate dipping in autoclaved and filtered seawater. Fragments (1-cm^3) of the specimen were aseptically cut and used for the isolation of bacteria while, few sponge fragments of the same size was immediately frozen on dry ice and stored at -80^0 C for DNA extraction.

***Bacterial Isolation, PCR and Sequencing*:** Sponge associated bacteria were isolated as described previously (Santavy *et al*. 1990). Approximately, 1g of the sponge tissue was aseptically removed and transferred to tube containing 99 ml of sponge dissociation medium, soaked for 20 min and homogenized. A tenfold serial dilution was made and spread plated on Zobell marine agar 2216 medium (HiMedia, Mumbai). All inoculated plates were incubated at ambient temperature (28 $\pm$ 1 $^{\circ}$C) for a week. Colonies developed on plates were enumerated on the seventh day and recorded as colony forming units (CFU). Bacteria showing distinct colony morphology were picked and purified on Zobell marine agar. The bacterial isolates were identified using a series of biochemical tests (Holt *et al*.2000; Bain and Shewan, 1968).

Molecular confirmation of the isolates was done by PCR amplification of the 16s rDNA gene using specific primers. Briefly, each purified isolate was suspended in 50 µl of colony lysis solution (10 mM Tris–HCl, pH 7.5, 10 mM EDTA and 50 µg/ml of proteinase-K) and incubated at 55 °C for 15 min followed by proteinase-K treatment at 80 °C for 10 min. The mixture was centrifuged at 15,000 x g at 4° C for 15 min and the supernatant containing the genomic DNA was used as template for PCR. Amplification of the 16S rDNA gene was carried out with eubacterial specific primers 27F (5'- AGA GTT TGA TCC TGG CTC AG -3') and 1492R (5'- GGT TAC CTT GTT ACG ACT T- 3'). PCR amplification was performed in a total volume of 50 µl reaction mixture consisting of 100 ng of template DNA, 1x PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 2.5 mM $MgCl_2$; 0.01% gelatin), 0.2 mM of dNTP mix, 10 pmol of each of forward and reverse primer, 1.5 U of *Taq* DNA polymerase (Bangalore GeNei, Bangalore). The PCR cycling conditions consisted of an initial delay at 94 °C for 10 min, followed by 35 cycles of 94 °C for 1 min, 54 °C for 2 min, and 72 °C for 2 min and a final extension step of 72 °C for 10 min. PCR products were separated by electrophoresis on a 1.5% (w/v) agarose gel, stained with 0.5 µg/ml ethidium bromide and bands were visualized under UV-transilluminator (Herolab, Germany).

Purification of the PCR products was done using QIAquick PCR purification kit (QIAGEN, Germany). The purified PCR products were cloned using the Strataclone® Cloing kit according to manufacturer's instructions (Strataclone, USA). The clones were sequenced using the M13 forward and reverse primers, ABI Big Dye Terminator Kit Version 3.1 and 3100 Genetic Analyser (PE Applied Biosystems, Foster City, California) by M/s. Bangalore GeNei, Bangalore.

Total DNA extraction from sponge, PCR and 16S clone library construction: Total community DNA was extracted from sponge tissue as described previously (Webster *et al*. 2001). Freeze dried sponge tissue (1.5g) was ground in liquid nitrogen and suspended in 5 ml of TE buffer (10 mM Tris-Cl and 1 mM EDTA, pH 8.0) containing 50 mg/ml lysozyme and incubated at 30 ^{0}C for 30 min. Guanidinium thiocyanate buffer (60% guanidinium thiocyanate, 20 ml of 100 mM EDTA and 5 ml of 10% Sarkosyl) was added and the mixture vortexed for 5 min. Samples were transferred to ice, and ammonium acetate was added to a final concentration of 2.5 M. The supernatant was transferred to a fresh tube and an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, vortexed and centrifuged at 10,000 × g for 10 min. The aqueous phase containing DNA was transferred to a fresh tube and precipitated by adding 2-3 volumes of isopropanol followed by centrifugation at 14,000 × g for 10 min. The pellet was washed with 1 ml of 95% ethanol and vacuum dried. Finally, the dried pellet was dissolved in 100 µl of 1× TE buffer (pH 8.0) and stored at -20 ^{o}C until further use. Two micro litres of the DNA extracted from marine sponge was taken for determining the DNA concentration using NanoDrop ND-1000 spectrophotometer (NanoDrop, USA).

PCR and clone library construction: PCR using eubacterial primers and cloning of purified products for sequencing was carried out by the protocol outlined above for culturable bacteria.

Phylogenetic analysis of 16S rDNA sequences: The partial 16S rDNA sequences were initially de-replicated by comparing all the sequences in a data set to each other, using the ALIGN program (http://blast.ncbi.nlm.nih.gov/bl2seq/wblast2.cgi) of NCBI. Only one representative sequence having ≥98% similarity was taken for further analysis. Sequences were also checked for chimeras using the program CHECK CHIMERA of the Ribosomal Database Project-II (RDP-II) website (http://rdp.cme.msu.edu/). Chimeric sequences were not included in the analysis. To identify known sequences with a high degree of similarity, the clone sequences were matched to sequences in the RDP-II database using the SEQMATCH program and with sequences in GenBank database using Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Based on the percentage similarity of unknown clone sequences to representative bacterial sequences in RDP–II and GenBank databases, the sequences were further classified up to their genus (93-96% match) and species (97-100% match) levels or considered as a related type (86-92% match) (Stackebrandt & Goebel, 1994). Phylogenetic and the neighbour joining phylogenetic trees were constructed using MEGA version 4 (Tamura *et al*. 2007). The robustness of inferred tree topologies was analysed by bootstrapping using 1000 replicates. The 16S rDNA sequence of *Fusobacterium* sp. (AY953256) was included as an out group to root the tree.

RESULTS

Culture-based studies: The bacterial population associated with *H. nigrocutis* was estimated to be 6.9 x 10^7 CFU/g. Based on morphological and biochemical characterisation, the cultivatable bacteria from *H.nigrocutis* were identified as *Vibrio, Bacillus* and *Acinetobacter* spp. Further, sequence analysis of 16S rRNA gene of representative morphotypes revealed the culturable bacteria to belong to two phylotypes: *Firmicutes* represented by *Bacillus* spp. with a sequence similarity of 90-99% and *Gamma-proteobacteria* represented by *Vibrio* spp. and *Acinetobacter* spp. having a sequence similarity of 91-98%. The phylogenetic affiliations of these isolates are presented in Fig. 1.

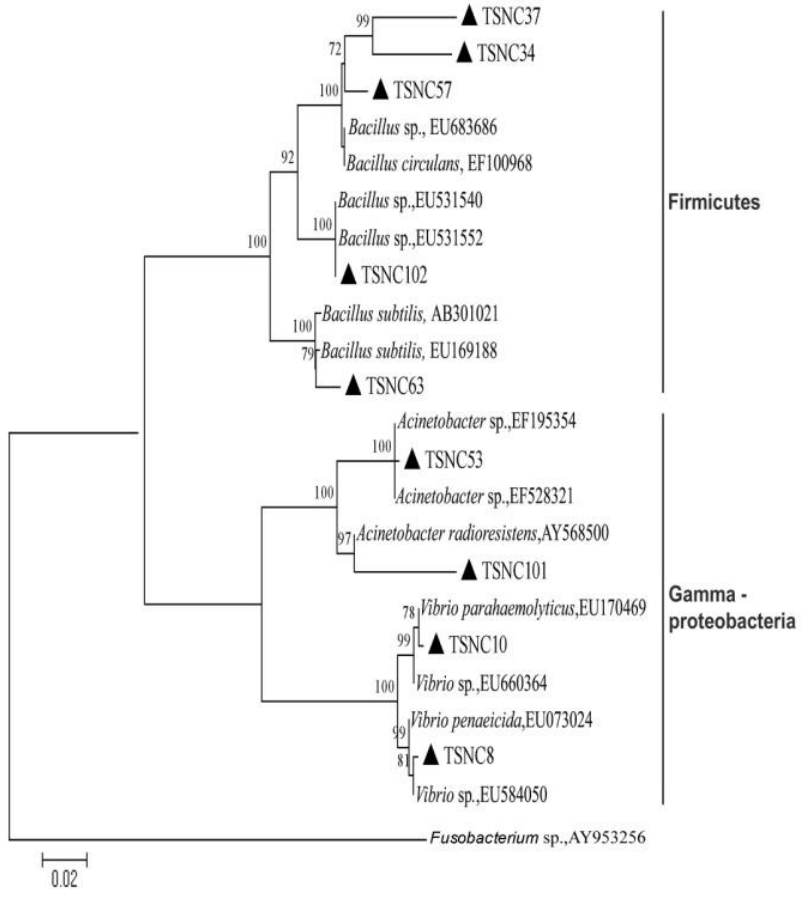

Fig. 1. Neighbour-joining phylogenetic tree constructed from analysis of 566 bp of 16S rDNA sequences of culturable bacteria from the marine sponge *Halichondria nigrocutis*. Numbers at the nodes are percentages indicating the bootstrap valves based on Maximum Composite Likelihood analysis from 1,000 replicates. Only values >50% are shown. The scale bar represents 0.02 substitutions per nucleotide position.

Phylogenetic analysis of 16S rDNA sequences: The unculturable bacteria associated with *H.nigrocutis* was investigated based on the 16S rDNA PCR yielding an amplicon of ~950 to 995 bp. 80 representative clones that were sequenced and analysed yielded 37 independent sequence profiles which affiliated the microbial sequences to 6 bacterial groups: *alpha-proteobacteria, gamma-proteobacteria, delta-proteobacteria, cyanobacteria, actinobacteria* and *firmicutes* (Fig. 2). The dominant species was represented by the *alpha-proteobacterial* group (n=25; 67.6%), followed by *gamma -proteobacteria*, *actinobacteria* and *firmicutes* (n=3; 8.1% each), *cyanobacteria* (n=2; 5.4%) and *delta-proteobacteria* (n=1; 2.7%). Most of the sequences obtained from *H.nigrocutis* were found to be closely related to bacterial sequences associated with sponges of distant taxonomic and geographical origin. Within the *alpha-proteobacterial* cluster a large number (n=16) of sequences were closely r elated (>95%) to

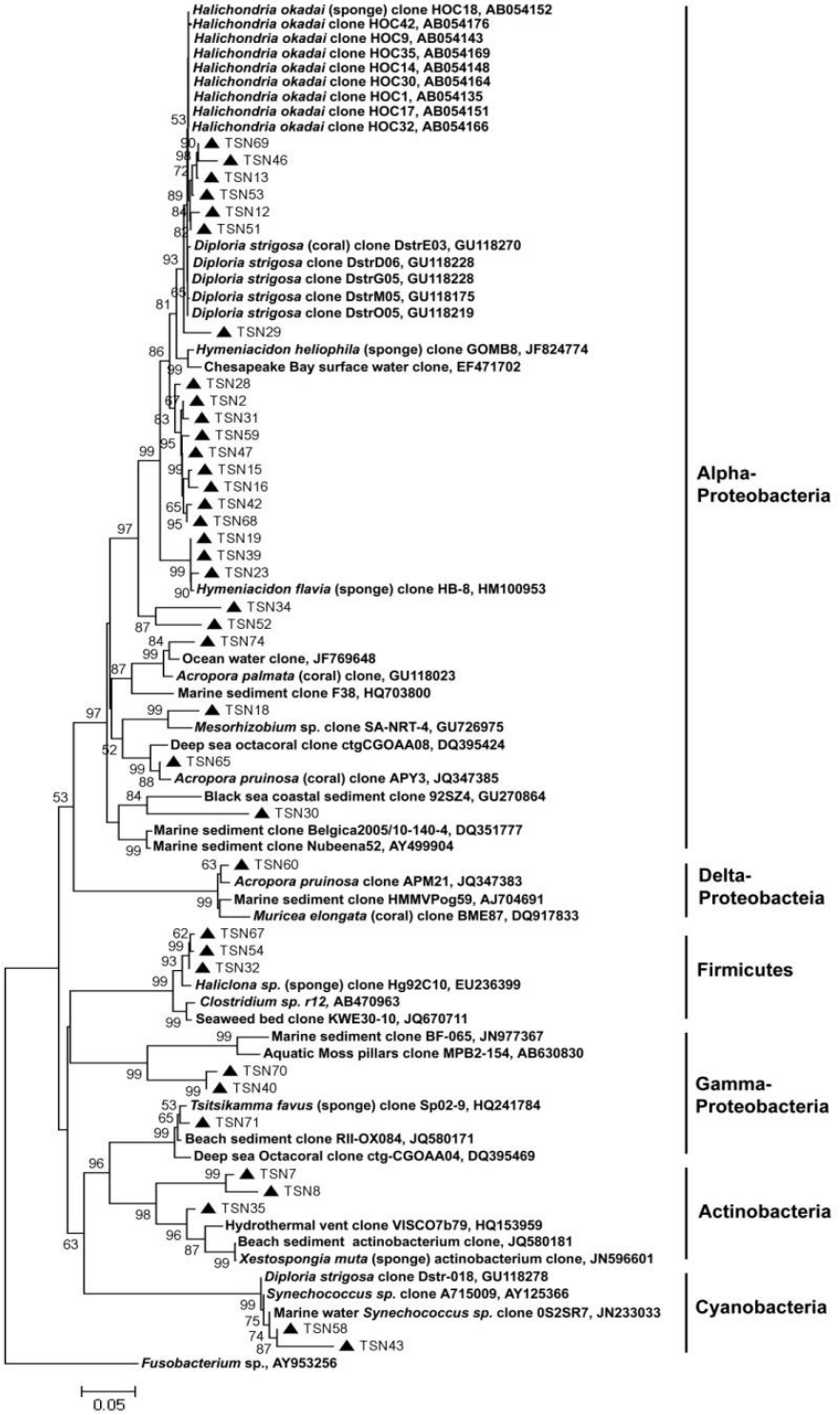

Fig. 2. Neighbor-joining phylogenetic tree from analysis of 773 bp of 16S rDNA sequences of clones in the library constructed from total DNA extracted from the marine sponge *Halichondria nigrocutis. H.nigrocutis* clone sequences are labeled as TSN followed by clone number. Numbers at the nodes are percentages indicating the bootstrap valves based on Maximum Composite Likelihood analysis from 1,000 replicates. Only values >50% are shown. The scale bar represents 0.05 substitutions per nucleotide position.

Table 1: *Halichondria nigrocutis* associated bacterial 16S rDNA clones and their closest match in GenBank

Clone	Accession no.	Nucleotides compared (bp)	Closest match in GenBank (% identity, accession number, sequence)
Alpha Proteobacteria			
TSN2	EU263911	967	97, AB054169, *Halichondria okadai* (sponge) bacterial clone HOC35
TSN12	EU263912	963	96, GU118228, *Diploria strigosa* (coral) bacterial clone DstrD06
TSN13	EU263913	974	98, GU118219, *Diploria strigosa* bacterial clone DstrD05
TSN15	EU263914	962	95, AB054166, *Halichondria okadai* bacterial clone HOC32
TSN16	EU263915	974	96, AB054164, *Halichondria okadai* bacterial clone HOC30
TSN18	EU812459	975	91, DQ351777, Marine sediment clone Belgica 2005/10-140-4
TSN19	EU302866	973	99, HM100953, *Haliclona permollis* (sponge) clone CC20
TSN23	EU302867	981	98, HM100953, *Haliclona permollis* clone CC20
TSN28	EU289145	973	97, AB054152, *Halichondria okadai* bacterial clone HOC18
TSN29	EU289146	949	94, GU118175, *Diploria strigosa* bacterial clone DstrM05
TSN30	EU302869	977	89, AY499904, Marine sediment clone Nubeena52
TSN 31	EU289147	973	96, AB054151, *Halichondria okadai* bacterial clone HOC17
TSN34	EU302868	964	89, HQ703800, Marine sediment clone F-38
TSN39	EU812460	973	94, JF824774, *Hymeniacidon heliophila* (sponge) bacterial clone 919-C8
TSN42	EU289148	962	95, AB054143, *Halichondria okadai* bacterial clone HOC9
TSN46	EU289149	974	97, GU118270, *Diploria strigosa* bacterial clone DstrE03
TSN47	EU289150	971	96, AB054135, *Halichondria okadai* bacterial clone HOC1
TSN51	EU289153	974	98, GU118219, *Diploria strigosa* bacterial clone DstrO05
TSN52	EU289154	978	91, AB054176, *Halichondria okadai* bacterial clone HOC42
TSN53	EU289151	980	98, GU118184, *Diploria strigosa* bacterial clone DstrG05
TSN59	EU289152	975	96, GU118228, *Diploria strigosa* bacterial clone DstrD06
TSN65	EU302870	973	98, JQ347385, *Acropora pruinosa* bacterial clone APY3
TSN68	EU289155	975	96, AB054169, *Halichondria okadai* bacterial clone HOC35
TSN69	EU289156	975	98, GU118228, *Diploria strigosa* bacterial clone DstrD06
TSN74	EU302871	955	96, GU118023, *Acropora palmata* (coral) bacterial clone Apal_D07
Gamma proteobacteria			
TSN40	EU302878	955	87, AB630830, Aquatic moss pillars clone MPB2-154
TSN70	EU302879	951	88, JN977367, Marine sediment clone B5-065
TSN71	EU302880	971	98, HQ241784, *Tsitsikamma favus* (sponge) clone Sp02-9
Delta proteobacteria			
TSN60	EU302877	985	97, JQ347383, *Acropora pruinosa* (coral) bacterial clone APM21
Actinobacteria			
TSN7	EU302881	976	85, JQ580181, Beach sediment actinobacterium clone
TSN8	EU302882	968	83, JN596601, *Xestospongia muta* (sponge) actinobacterium clone
TSN35	EU302883	972	92, HQ153959, hydrothermal vent filamentous microbial mat clone VISCO7b79
Cyanobacteria			
TSN43	EU302875	957	95, JN233033, Marine water *Synechococcus* sp clone 0S2SR7
TSN58	EU302876	975	99, AY125366, *Synechococcus* sp clone A715009
Firmicutes			
TSN32	EU302874	970	97, AB470963, *Montipora sp*. (coral) *Clostridium sp. R12*
TSN54	EU302872	970	94, EU236399, *Haliclona sp.* (sponge) bacterial clone Hg92C10
TSN67	EU302873	962	97, JQ670711, Sea weed bed clone KWE30-10

alphaproteobacterial symbionts associated with the marine sponge, *H.okadai* and the marine coral *Diploria strigosa*. The *alpha-proteobacterial* clones TSN19, TSN23 and TSN39 had 98-99% affiliation to the marine sponge *Hymeniacidon flavia* bacterial clone, HM100953. Clone TSN65 was closely affiliated to the clones associated with the Cnidarian *Acropora pruinosa* (98%) and Deep sea octacoral (95%). Among the *gamma-proteobacteria*, the clone TSN71 matched closest to clone HQ241784 associated to host *Tsitsikamma favus*, a marine sponge endemic to the coast of South Africa. The *Delta-proteobacteria* sequence (TSN60) had affiliation (97-98%) with the unculturable proteobacterial clones associated with Cnidaria *Muricea elongata* and *Acropora pruinosa*. *Cyanobacterial* clones (TSN43, TSN58) had close species level affiliations to uncultured *Synechococcus* sp. from oligotrophic oceanic waters and to a clone associated with the coral *D. strigosa*. *Actinobacteria* clone (TSN35) showed near complete identity (99%) to uncultured actinobacterium clones derived from marine eukaryotes, water and sediments. *Firmicutes* (TSN32, TSN54 and TSN67) had sequence similarity (>94%) with a culturable *Clostridium* clone (AB470963) associated with a *Montipora* a marine coral species. Considering the criterion that sequences with <93% similarity to already available bacterial sequences are used for the proposal of a new genus and >97% for the proposal of a new species (Stackebrandt and Goebel, 1994), 4 *alpha-proteobacterial* (TSN18, TSN30, TSN34, TSN52), 2 *gamma-proteobacterial* (TSN40, TSN70) and 2 actinobacterial (TSN7, TSN8) clones associated with *H.nigrocutis* were seen to be novel at the genus level while the remaining could be considered novel at the species level (Table 1). The sequences have been submitted to GenBank and assigned accession numbers (Table 1).

DISCUSSION

This is the first study of the bacterial communities associated with the marine sponge *H.nigrocutis*. The bacteria associated with *H.nigrocutis* included those belonging to the phyla *Proteobacteria* (*alpha-, delta- and gamma*-classes), *Cyanobacteria*, *Firmicutes* and *Actinobacteria*. The largest cluster was represented by the *Proteobacterial* group which is consistent with data derived from other sponge-associated 16S rRNA gene libraries (Webster and Taylor 2012). Among the uncultured *Proteobacterial* group associated with *H.nigrocutis*, *alpha-proteobacteria* was the most predominant, followed by Gamma- and Delta proteobacteria. Proteobacterial groups (*alpha-*, *beta-*, *gamma-*, *delta*-classes) have been reported for several sponge species such as *Aplysina cavernicola* (Thomas *et al*. 2003), *Rhopaloeides odorabile* (Webster *et al*. 2001; Webster and Hill 2001), *Theonella swinhoei*, (Schmidt *et al*. 2000) and *H.panacea* (Wichels *et al*. 2006). Interestingly, most of the *alpha-proteobacteria* sequences analysed in this study were closest to uncultured *alpha-proteobacterial* clones associated with the Japanese marine sponge *H. okadai* and the Caribbean coral *Diploria strigosa.* The results are in agreement with an earlier study by Taylor *et al*. (2007) and a recent analysis by Sunagawa *et al*. (2010) wherein they report a number of monophyletic 16S rRNA clusters to contain exclusively sponge- and coral-derived sequences. The culturable bacterial community associated with *H.nigrocutis* consisted of *Firmicutes* and *Gamma-proteobacteria*.

Recent studies have shown that *Firmicutes* (low G+C Gram positive bacteria) are identified from marine sponges by both cultivation-dependent and cultivation-independent approaches (Webster and Taylor 2012). Based on analysis of denaturing gradient gel electrophoresis, the main cultivable group within the phylum *Firmicutes* is reported to be the *Bacillus* spp. (Taylor *et al*. 2007). Even in the marine sponges *Aplysina cavernicola* (Hentschel *et al*. 2001), *S. zeteki* (Zhu *et al*. 2008), *Haliclona simulans* (Phelan *et al*. 2011) and *H.panicea* (Imhoff and Stohr, 2003) the predominant genus of culturable bacteria reported was *Bacillus* spp. Marine *Bacillus* spp. has been recognised as a source of many antibiotics such as cyclic peptides, cyclic lipopeptides and novel thiopeptides (Nagai *et al*. 2003). Interestingly, spore formation in *Bacillus* spp. has been shown to be co-regulated with antibiotic production (Marahiel *et al*. 1993). We surmise that, the association of *Bacillus* and *Clostridium* spp. with *H.nigrocutis* is probably a mutualistic one wherein these bacteria may be playing a critical role in sponge defence against colonization by harmful organisms as also reported earlier (Thakur *et al*. 2004). Likewise, the association of *Acinetobacter* and *Vibrio* spp. with *H.nigrocutis* is of great interest, because several species of these genera associated with sponges are known to be secondary metabolite producers (Elyakov *et al*. 1991; Oclarit *et al*. 1994) and are a recognized source of natural products.

Actinobacteria originally isolated from soil are now being increasingly isolated from seawater and marine sediments. They are well known as producers of secondary metabolites with high pharmacological and commercial application (Jensen *et al*. 2007; Taylor *et al*. 2007; Gontang *et al*. 2010). Sponge-derived actinobacteria have been reported from marine sponges such as *Theonella swinhoei, A. aerophoba* (Hentschel *et al*. 2001) *Rhopaloeides odorabile* (Webster *et al*. 2001), *Xestospongia* spp. (Montalvo *et al.* 2005), *Chondrilla nucula* (Thiel *et al.* 2007) and *Hymeniacidon perleve* (Zhang *et al*. 2006) and therefore the association of actinobacteria clones with the sponge *H.nigrocutis* is noteworthy and demands further study. The *Cyanobacteria* are known to be associated as photosynthetic symbionts with the marine sponges commonly found in shallow water marine environments (Usher *et al*. 2001; Thacker and Freeman, 2012) wherein both are mutually benefitted. Cyanobacteria has been previously reported to be found in 26 *Demospongiae* families (Hentschel *et al*. 2006) and the association of cynobacterial clones in the host *H. nigrocutis,* although a minor component of the clone library, is in agreement with this. Marine *Cyanobacteria* belonging to the *Synechococcus* species has been shown to predominate the nutrient-rich gyres of tropical oceanic waters (Moore *et al*.1995) and implicated in the uptake and utilization of organic nitrogen compounds as a nitrogen source (Moore *et al*. 2002; Zubkov *et al*. 2003). The cynobacterial clones in this study (TSN43, TSN68), was closely related to an uncultured *Synechococcus* clone (AY125366) shown to be involved in uptake of organic nitrogen in oligotropic oceanic waters (Zubkov *et al*. 2003). Therefore, it needs to be seen whether the association of *Cyanobacteria* associated with *H.nigrocutis* is truly symbiotic and contributes to any beneficial effects to the host in serving as a source of nutrition.

In this study, surveys of associated microbial communities using the 16S rRNA resulted in the revelation of many novel bacterial species previously not known, within the sponge *H.nigrocutis.* As the unique bacteria–sponge relationship has been a subject of intense research due to marine organisms being a source of natural products, the novel sequences associated with this sponge await future exploration for elucidating the specific factors involved in sponge–microbe interactions, the functional nature and the putative benefits of these interactions.

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