RESEARCH ARTICLE

Molecular richness and biotechnological potential of bacteria cultured from Irciniidae sponges in the north-east Atlantic

Ana I.S. Esteves[1], Cristiane C.P. Hardoim[1], Joana R. Xavier[2,3], Jorge M.S. Gonçalves[4] & Rodrigo Costa[1]

[1]Microbial Ecology and Evolution Research Group, Centre of Marine Sciences, Universidade do Algarve, Faro, Portugal; [2]CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, InBIO Laboratório Associado, Pólo dos Açores – Departamento de Biologia da Universidade dos Açores, Ponta Delgada, Portugal; [3]CEAB, Centre d'Estudis Avançats de Blanes, (CSIC), Blanes (Girona), Spain; and [4]Fisheries, Biodiversity and Conservation Research Group, Centre of Marine Sciences, University of Algarve, Faro, Portugal

Correspondence: Rodrigo Costa, Centre of Marine Sciences, University of Algarve, Gambelas Campus, Building 7, Room 2.77, 8005-139 Faro, Portugal.
Tel./fax: (+351) 289 800 051;
e-mail: rscosta@ualg.pt

Received 16 January 2013; revised 19 April 2013; accepted 19 April 2013.
Final version published online 20 May 2013.

DOI: 10.1111/1574-6941.12140

Editor: Angela Sessitsch

Keywords
marine sponges; antimicrobial activity; bacterial diversity; genotyping.

Abstract

Several bioactive compounds originally isolated from marine sponges have been later ascribed or suggested to be synthesized by their symbionts. The cultivation of sponge-associated bacteria provides one possible route to the discovery of these metabolites. Here, we determine the bacterial richness cultured from two irciniid sponge species, *Sarcotragus spinosulus* and *Ircinia variabilis*, and ascertain their biotechnological potential. A total of 279 isolates were identified from 13 sponge specimens. These were classified into 17 genera – with *Pseudovibrio*, *Ruegeria* and *Vibrio* as the most dominant – and 3 to 10 putatively new bacterial species. While 16S rRNA gene sequencing identified 29 bacterial phylotypes at the 'species' level (97% sequence similarity), whole-genome BOX-PCR fingerprinting uncovered 155 genotypes, unveiling patterns of specimen-dependent occurrence of prevailing bacterial genomes across sponge individuals. Among the BOX-PCR genotypes recovered, 34% were active against clinically relevant strains, with *Vibrio* isolates producing the most active antagonistic effect. Several *Pseudovibrio* genotypes showed the presence of polyketide synthase (PKS) genes, and these were for the first time detected in isolates of the genus *Aquimarina* (*Bacteroidetes*). Our results highlight great biotechnological potential and interest for the Irciniidae sponge family and their diversified bacterial genomes.

Introduction

In so-called high microbial abundance (HMA) sponges, as much as 38% of animal biomass is attributed to prokaryotes (Vacelet & Donadey, 1977), exceeding numbers found in seawater by 2–4 orders of magnitude (Hentschel *et al.*, 2006). The capability of hosting such abundant microbial communities has prompted scientists to suggest the Porifera as one of the most – if not the most – microbial-permissive, extant animal phyla (Schmitt *et al.*, 2007). Since the inaugural studies on sponge-associated bacteria (Vacelet, 1975; Vacelet & Donadey, 1977), the development of several molecular biology techniques and use of high-throughput DNA sequencing methods have enabled novel insights into the diversity and function of the marine sponge microbiome (Webster *et al.*, 2010; Lee *et al.*, 2011; Fan *et al.*, 2012; Schmitt *et al.*, 2012; Simister *et al.*, 2012). Among the plethora of microorganisms in the three domains of life that may inhabit these animals, bacteria conspicuously emerge as the most dominant and diverse (Taylor *et al.*, 2007). In spite of the severe limitations that hamper their experimental verification, several roles have been hypothesized for bacteria inhabiting marine sponges. These include involvement in sponge nutrient metabolism and elemental cycling (Thomas *et al.*, 2010; Webster & Taylor, 2012), host chemical defence by bioactive secondary metabolites production (Piel *et al.*, 2004; Hochmuth & Piel, 2009) and sponge structural rigidity (Wilkinson, 1978), among others (reviewed by Taylor *et al.*, 2007; Hentschel *et al.*, 2012 and Webster & Taylor, 2012). Resident sponge bacteria are in their turn thought to benefit from diverse and

abundant nutritional factors and shelter within sponges (Taylor *et al.*, 2007; Hentschel *et al.*, 2012).

Marine sponges are regarded as the most relevant reservoir of biologically active metabolites in the seas (Piel, 2004), with more than 280 new structures reported in 2010 and similar numbers in previous years (Blunt *et al.*, 2012). With growing evidence microbial symbionts rather than the host itself might in fact produce several of the documented sponge-derived bioactive compounds (Piel, 2004; Hentschel *et al.*, 2012). Polyketides and nonribosomal peptides are often evoked as examples of metabolites found in sponges with a likely (bacterial) symbiont origin. These molecules, synthesized by large multifunctional enzymes called polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS), encompass substance classes that are typical for microorganisms (Piel, 2004; Fisch *et al.*, 2009). They possess intricate and diverse structures that display a wide range of relevant pharmaceutical bioactivities including antitumoral, antifungal and antiparasitic (Staunton & Weissman, 2001; Finking & Marahiel, 2004; Hochmuth & Piel, 2009).

Cultivation-independent approaches such as metagenomics and single-cell genomics have been of utmost relevance for the discovery of novel biosynthetic gene clusters – including PKS and NPRS operons – from recalcitrant or hard-to-cultivate sponge symbionts (Piel *et al.*, 2004; Fisch *et al.*, 2009; Siegl & Hentschel, 2010; Siegl *et al.*, 2011; Bayer *et al.*, 2013). Cultivation of microorganisms, in spite of its acknowledged limitations in recovering symbiont communities with fidelity, remains an attractive and essential endeavour for microbial biodiscovery, ecophysiology and evolutionary research. It allows full laboratory experimentation, delivering readily available biomass and complete genomes from accurately identified sources (Giovannoni & Stingl, 2007; Joint *et al.*, 2010). Culturing might also be fundamental to our understanding of sponge microbiome diversity, as cultivation-independent studies typically rely on analyses of single phylogenetic markers such as 16S rRNA genes, which intrinsically prohibit species- and subspecies-level assessments of diversification within this complex microbiota. In this regard, PCR-based techniques that explore repetitive elements present across the bacterial genome – such as BOX-PCR targeting the highly conserved repeat BOX element (Martin *et al.*, 1992) – enable assessment of genotypic variation at the subspecies and strain levels (reviewed by Ishii & Sadowsky, 2009) and might constitute an excellent tool in diversity surveys of sponge symbiont communities. Finally, bacteria cultured from, for example, *Ircinia variabilis* (De Rosa *et al.*, 2003), *I. muscarum* (Mitova *et al.*, 2003) and *Suberites domuncula* (Mitova *et al.*, 2004) have been shown to synthesize novel cyclic peptides of putative nonribosomal origin and/or displaying antimicrobial activities. This highlights the potential of culturing symbiotic bacteria for secondary metabolite research and their corresponding bioactivities.

This is the first extensive study of bacteria cultured from Irciniidae sponges (Demospongiae, Dictyoceratida) in the North Atlantic. Species in this family are nonspiculated HMA sponges (Vacelet & Donadey, 1977; Hentschel *et al.*, 2006), with a dense mesohyl layer (Schmitt *et al.*, 2007; Weisz *et al.*, 2008) and a skeleton composed of primary and secondary spongin fibres and collagen filaments (Cook & Bergquist, 2002). The family embraces the genera *Ircinia*, *Sarcotragus* and *Psammocinia*. Particularly, *Ircinia* and *Sarcotragus* species are abundant across the Atlanto-Mediterranean zone and along the Algarvian coast, south Portugal (Pires, 2007). They have often been reported as sources of novel metabolites belonging to several chemical classes and presenting manifold bioactivities (De Rosa *et al.*, 1996, 1997; Rifai *et al.*, 2005a,b; Liu *et al.*, 2006, 2008; Shen *et al.*, 2006, 2009; Wang *et al.*, 2008), but the antagonistic potential of their symbionts remains understudied. Here, we contrast the assemblage of bacterial isolates retrieved from the species *Sarcotragus spinosulus* Schmidt, 1862 and *Ircinia variabilis* Schmidt, 1862, in terms of richness, diversity and composition. To this end, a fine-tuned genomics analysis including whole-genome typing beyond 16S rRNA gene sequencing and phylogeny was employed. To address the biotechnological potential of these isolates, especially concerning their antimicrobial activities, their genomes were screened for polyketide synthase and nonribosomal peptide synthase genes, and their *in vitro* antagonistic activity towards clinically relevant bacteria was determined.

Materials and methods

Isolation and cultivation of sponge-associated bacteria

A total of 13 sponge specimens recognized *in situ* as members of the family Irciniidae were collected in June 2010 by scuba diving, along the southern coast of Portugal, at Galé Alta (37°04′09.6N, 8°19′52.1W) at *c.* 15 m depth. Each sponge specimen was placed individually, immersed in surrounding seawater, in a sterile zip-lock bag. Each bag was kept refrigerated inside a cooling container and brought to the laboratory, where sponges were processed immediately. A fragment of each sponge specimen was excised and preserved in 90% ethanol for classical and molecular taxonomic identification, revealing that nine specimens belonged to *S. spinosulus* and four to *I. variabilis* (see Hardoim *et al.*, 2012 for procedures). These vouchers were deposited in the Biology Department's zoological collection of the University of the Azores (DBUA.Por). For bacterial cultivation, sponge

specimens were rinsed with artificial seawater (ASW: 23.38 g L^{-1} NaCl, 2.41 g L^{-1} $MgSO_4$, 1.90 g L^{-1} $MgCl_2$, 1.11 g L^{-1} $CaCl_2$, 0.75 g L^{-1} KCl and 0.17 g L^{-1} $NaHCO_3$), and 2.5 g of sponge was cut and macerated in 25 mL of ASW using a sterile mortar and pestle. The supernatant was transferred to a falcon tube, and the remaining sponge residue was discarded. A dilution series was prepared in ASW, and 100 μL of dilutions 10^{-3} to 10^{-5} was plated on sterile marine agar (MA: 40.1 g L^{-1} marine broth by ROTH® and 15 g L^{-1} agar) in triplicate. Plates were incubated for 3 days at 25 °C. To allow for maximum richness coverage, colony forming units (CFUs) with different morphologies (usually 3–4 colony morphotypes per specimen) were first searched, and further CFUs were picked at random until a number of 25 CFUs per specimen were achieved. CFUs were then purified by successive streaking on new MA plates. Pure isolated colonies were transferred to sterile liquid medium (40.1 g marine broth in 1 L) and allowed to grow for 48 h at room temperature with shaking (200 rpm). An aliquot of each liquid culture was taken and stored in 20% glycerol at −80 °C until further use. Two millilitres of the remaining culture was centrifuged at 10 000 ***g*** for 5 min, the supernatant was discarded, and the bacterial pellet was stored at −20 °C for DNA extraction.

Bacterial DNA extraction and identification

DNA was extracted from previously stored bacterial pellets with the Wizard Genomic DNA Purification kit (Promega Corporation, Madison, WI), according to the manufacturer's instructions. Genus-level identification of isolates was determined by 16S rRNA gene sequencing. 16S rRNA gene fragments of *c.* 1500 bp in length were amplified using the bacterial universal primers F27 (5′-AGA GTT TGA TCM TGG CTC AG-3′) and R1492 (5′-TAC GGY TAC CTT GTT ACG ACT T-3′) (Weisburg *et al.*, 1991). Reaction mixtures (25 μL) were prepared as follows: 2.5 μL of 10× BIOTAQ DNA Polymerase NH_4 Buffer, 1.88 μL of $MgCl_2$ 50 mM, 1.25 μL BSA 2 mg mL^{-1}, 0.5 μL DMSO 100%, 2.5 μL dNTPs 2 mM, 0.4 μL of each primer 10 μM, 0.125 μL of BIOTAQ DNA Polymerase (Bioline, London, UK) 5 U μL^{-1} and 1 μL (*c.* 50 ng) of template DNA. Thermal cycling started with an initial denaturation step of 94 °C for 5 min, 25 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 45 s and a final extension step of 72 °C for 10 min.

All PCR amplifications were carried out in a MyCycler thermal cycler (Bio-Rad, Hercules, CA). All amplicons were checked under UV light after electrophoresis in 1% agarose gels stained with 1× GelRed (Biotium, Hayward, CA). PCR products with the right size (*c.* 1500 bp) were cleaned with Sephadex G50 (GE Healthcare Bio-Science AB, Uppsala, Sweden) columns, quantified with IMAGE LAB™ Software (Bio-Rad) and subjected to sequencing with the chain termination method in an Applied Biosystems 3130 genetic analyser using the forward primer. Nearly complete 16S rRNA gene sequences were obtained for putatively novel bacterial species – as indicated by preliminary partial sequence analysis – by sequencing with the forward and reverse primers. All sequences were manually trimmed using Sequence Scanner v1.0 software (Applied Biosystems), assembled using DNA BASER 3.5.0 and checked for chimera formation using PINTAIL version 1.0 (Ashelford *et al.*, 2005). Their closest phylogenetic relatives were searched using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) database. Taxonomic assignment of bacterial isolates to the genus level was performed using the classifier tool of the Ribosomal Database Project (RDP, release 10, http://rdp.cme.msu.edu) at 80% confidence threshold. Closest type strains to all sequence queries were determined using the RDP sequence match tool. 16S rRNA gene sequences were deposited in the EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk/ena/) under accession numbers HE818111–HE818389.

16S rRNA gene richness and diversity

Evolutionary distances between 16S rRNA gene sequences were calculated with the Kimura 2-parameter and applied to generate pairwise similarity matrices with the DNADIST software (http://cmgm.stanford.edu/phylip/dnadist.html). These were used as templates for the assignment of sequences to operational taxonomic units (OTUs) using the furthest-neighbour method as implemented in the DOTUR software (Schloss & Handelsman, 2005). The frequency data assigned to 'unique' OTUs – defined at 99% and 97% levels of similarity – were employed for the construction of rarefaction curves and estimation of theoretical richness using the Chao1 estimator. To determine whether bacterial collections derived from specimens identified as *I. variabilis* and *S. spinosulus* were significantly different in their composition, library shuffling analysis using the program MOTHUR was performed (Schloss *et al.*, 2009).

Phylogenetic analysis

For phylogenetic inference, all 16S rRNA gene sequences were aligned using the SINA web aligner (Pruesse *et al.*, 2007) and imported into the SILVA 16S rRNA database version 102 using the parsimony tool as implemented in the ARB software (Ludwig *et al.*, 2004). Alignments were manually refined using the ARB alignment tool. The 16S rRNA gene sequences of closest matches observed in BLAST

analysis were included in the alignment procedure. An appropriate evolutionary model was then determined using MRMODELTEST (vers. 2.3) (Nylander, 2008). This was the general-time reversible model (GTR) (Rodríguez *et al.*, 1990) with a discrete gamma-distribution of among-site rate variation (Γ_4) and a proportion of invariant sites (I). An optimal maximum-likelihood tree was determined using RAXML (vers. 7.0.4-MPI, Stamatakis, 2006) with 100 replicates, each starting from a random tree, with the GTR+Γ_4+I model. Maximum-likelihood bootstrap support was determined with the same software and model using 300 replicates with thorough final optimization of the tree from each replicate. A Bayesian MCMC analysis was conducted using MRBAYES (vers. 3.2.1, Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) each of two runs using four chains (Metropolis coupling) for 2 million generations, sampling every 1000 generations, and using the GTR+Γ_4+I model.

Whole-genome bacterial fingerprinting (BOX-PCR)

To evaluate the extent of whole-genome diversification within isolates, repetitive BOX element PCR-based genotyping (BOX-PCR) was performed using the BOX A1R single primer (5′-TA CGG CAA GGC GAC GCT GAC G-3′) (Versalovic *et al.*, 1994). Amplification reactions were carried out in a 25 μL mix containing 2.5 μL of BIOTAQ NH_4 buffer 10×, 1.25 μL of $MgCl_2$ 50 mM, 1.25 μL DMSO 100%, 2.5 μL dNTPs 2 mM, 0.5 μL of primer 10 μM, 0.5 μL of BIOTAQ DNA Polymerase 5 U μL^{-1} and 2 μL of template DNA. Amplification conditions consisted of an initial denaturation step of 95 °C for 5 min, 10 cycles of 94 °C for 10 s, 52 °C for 1 min, 68 °C for 4 min, followed by 25 cycles of 94 °C for 10 s, 52 °C for 1 min, 68 °C for 10 s with sequential 10-s increments and a final extension step of 65 °C for 16 min (Currie *et al.*, 2007). Products (12 μL) were loaded on 1% agarose gels, electrophoresed at 110 V for 200 min, stained with GelRed 1× and photographed under UV light using Image Lab™ Software. Cluster analysis of BOX-PCR profiles was performed with GelCompar II version 6.5 (Applied Maths, Kortrijk, Belgium) using Pearson correlation indices of similarity with the unweighted pair-group method using arithmetic averages (UPGMA). A cut-off level of similarity was used to determine whether isolates shared the same BOX-PCR profile. This value was calculated for each bacterial genus separately by comparing several profiles of one representative isolate of each genus, with such profiles being generated by independent BOX-PCR amplifications, which were then loaded on multiple agarose gels. The Shannon measure of diversity (H′), determined as $H' = -\sum \Pi \log \Pi$, where *pi* represents the relative abundance of the 'ith' category within the sample, was applied to estimate genotypic diversity of whole-genome bacterial fingerprints.

Screening for polyketide synthase type I (PKS) and nonribosomal peptide synthetase (NRPS) genes

For type I PKS gene screening, the KS (ketosynthase) gene fragments were amplified using the inosine-based degenerate primers degKS2F.i (5′-GCI ATG GAY CCI CAR CAR MGI VT-3′) and degKSR5.i (5′-GTI CCI GTI CCR TGI SCY TCI AC-3′) (Schirmer *et al.*, 2005). For NRPS gene screening, the inosine-based degenerate primers degNRPS-1F.i (5′-AAR DSI GGI GSI GSI TAY BIC C-3′) and degNRPS-4R.i (5′-CKR WAI CCI CKI AIY TTI AYY TG-3′) amplified the adenylation domain gene fragments (Schirmer *et al.*, 2005). A reaction volume of 50 μL contained 5 μL of BIOTAQ NH_4 buffer 10×, 3.75 μL of $MgCl_2$ 50 mM, 5 μL DMSO 100%, 5 μL dNTPs 2 mM, 2 μL of each primer 10 μM, 0.25 μL of BIOTAQ DNA Polymerase 5 U μL^{-1} and 1 or 2 μL (50–100 ng) of template DNA. Amplification cycles consisted of an initial denaturation step of 94 °C for 5 min, 40 cycles of 94 °C for 40 s, 44 °C for 40 s, 72 °C for 75 s (or 105 s for NRPS amplification) and a final extension step of 72 °C for 10 min. Amplicons were visualized, cleaned and directly sequenced as described above for 16S rRNA gene amplifications. A positive PCR signal was recorded for amplicons of the right size (700 bp and 1000 bp for PKS and NRPS genes, respectively) showing no side bands. Only high-quality gene sequences retrieved from such amplicons were further analysed and deposited in the EMBL Nucleotide Sequence Database under accession numbers HE818089–HE818110 and HF968475-HF968492.

Antagonism assays

Antagonistic activity was assessed for single genotype isolates using a double-layer method performed as described elsewhere (O'Halloran *et al.*, 2011) with minor alterations. Isolates were grown for one week in marine broth at room temperature (20–24 °C). Five microlitres of each stationary phase culture was spotted on MA plates and incubated at room temperature for 2 days. Colonies were then overlaid with tryptic soy broth (TSB) soft agar (30 g L^{-1} TSB and 7.5 g L^{-1} agar) seeded with an overnight culture of the indicator strain being tested. Indicator strains *Staphylococcus aureus* NCTC 6571 (Gram-positive) and *Escherichia coli* NCTC 9001 (Gram-negative) were grown in TSB at 37 °C. Plates were incubated overnight at 37 °C, and presence (+) or absence (-) of growth inhibition of the indicator strains were recorded.

Results

Taxonomic classification

A total of 327 bacterial isolates were retrieved from 13 sponge specimens: 227 bacteria isolated from nine specimens of *S. spinosulus* and 100 bacteria isolated from four specimens of *I. variabilis*. Nearly complete 16S rRNA gene amplification and subsequent partial gene sequencing were successful for 279 isolates, with 188 amplicons (82%) obtained for bacteria isolated from *S. spinosulus* and 91 (91%) from *I. variabilis*. The number of bacterial isolates characterized from each sponge specimen was relatively even and ranged from 16 in the least sampled specimen to 24 in the most sampled specimens, averaging 21.5 ± 2.2 (standard deviation) isolates per specimen. Isolated bacteria were distributed among four bacterial phyla – *Actinobacteria* (0.4%), *Firmicutes* (0.4%), *Bacteroidetes* (5.4%) and *Proteobacteria* (93.9%) – with a clear dominance of the latter in both sponge species. Within the *Proteobacteria* phylum, the *Alphaproteobacteria* was the most abundant class in the two Irciniidae sponges (75.6% in *I. variabilis* and 65.3% in *S. spinosulus*). Isolated bacteria fell into 17 different genera, with *Pseudovibrio*, *Ruegeria* and *Vibrio* being the most abundant (Table 1). Notably, these dominant genera were also widespread throughout the surveyed sponge specimens, with *Pseudovibrio* isolates occurring in all 13 samples, followed by *Ruegeria* (registered in 11 of 13 samples) and *Vibrio* isolates (10 in 13).

Comparative analysis of genotypic richness, diversity and composition

Molecular richness, diversity and composition of cultured bacteria were assessed at four distinct levels of genotypic resolution. Using the complete 16S rRNA gene libraries from both sponge species, taxon-centred analyses were performed employing (1) genus-level classification of isolates and sequence similarity thresholds of (2) 97 and (3) 99% for the determination of bacterial operational taxonomic units (OTUs). In addition, (4) whole-genome genotyping using the BOX-PCR methodology was carried out to determine the extent of genome-wide clonality and diversification within isolates, especially those classified in the same genus and sharing high 16S rRNA gene homologies. Also, because of the effect that libraries of different size have on richness and diversity estimations, and on community composition assessments, comparative analyses with normalized sample sizes ($n = 91$), using *S. spinosulus*-derived sequences taken at random, were performed. Taking all sequences retrieved in this study into account, higher bacterial richness at the genus level was retrieved for *S. spinosulus*, with 15 different bacterial genera against nine genera found in *I. variabilis* (Fig. 1a). Although the average number of bacterial genera retrieved per host specimen was still higher in *S. spinosulus* (5.33 ± 0.71), it did not significantly differ from that registered in *I. variabilis* (4.75 ± 0.75) using a Student's t-test for independent samples ($t = 0.492$, *d.f.* = 11, $P = 0.632$). The richness of bacterial genera dropped from 15 to 11 in the normalized *S. spinosulus* library, as the low abundance taxa *Amphritea, Colwellia*, *Roseovarius* and *Shewanella* were lost in this sample. The relative abundances of dominant and moderately dominant genera were, nevertheless, maintained in the normalized *S. spinosulus* library (± 5% deviations in comparison with the full library). Remarkable differences in genus-level composition between the bacterial culture libraries consisted of (1) a higher incidence of *Shewanella* isolates retrieved from *I. variabilis* (12% of hits in library) than from *S. spinosulus* (2% of hits in the full library) and (2) an almost exclusive occurrence of *Microbulbifer* isolates in *S. spinosulus* (16 sequences, 9% of hits in the full library) (Fig. 1a). Library shuffling analysis revealed that 16S rRNA gene-based community composition differed between *Sarcotragus* and *Ircinia* at 99% and 97% sequence similarity thresholds for both full and normalized libraries comparisons. The full *S. spinosulus* library was found to cover the diversity present in the *I. variabilis* library ($P = 0.005$ at 99% cut-off; $P = 0.011$ at 97% cut-off), whereas the opposite was not true ($P > 0.05$). For normalized sample sizes, however, each of the libraries was found to contain residual diversity that could not be covered by the other library ($P < 0.02$ in all comparisons). Accordingly, rarefaction curves generated with full libraries depicted higher 16S rRNA gene richness in *S. spinosulus* than in *I. variabilis* (Fig. 1b). Here, the rarefied richness of bacterial isolates in *S. spinosulus* specimens tended to reflect that observed for both libraries together, whereas the same was not true for the *I. variabilis* library (Table 2). Overall, at 99% gene similarity threshold, 36 different 16S rRNA gene OTUs were found, 15 OTUs in *I. variabilis* and 28 OTUs in *S. spinosulus* (Table 2), with only seven OTUs (19.4% of total) shared between both sponge species. This trend was maintained when comparisons were made at a 97% cut-off level for the determination OTUs (Table 2), whereby *I. variabilis* and *S. spinosulus* shared only 9 from a total of 29 OTUs, with 5 and 15 OTUs found to be exclusive to each species, respectively. Using the randomized sampling of 91 sequences from *S. spinosulus*, this species was found to host 17 and 14 bacterial OTUs at 99% and 97% cut-off thresholds, respectively, displaying richness and diversity measures comparable with those obtained for *I. variabilis* (Table 2). BOX-PCR fingerprinting revealed that 155 isolates effectively represented different genotypes, with 113 genotypes found in *S. spinosulus* (*c.* 60% of the isolates representing a distinct genotype) and 49 genotypes in *I. varia-*

Table 1. Genus-level classification and genotype richness of bacteria isolated from *I. variabilis* and *S. spinosulus*

Phylum	Class	Order	Family	Genus*
Firmicutes 1 isolate 1 genotype	*Bacilli*	*Bacillales*	*Bacillaceae*	*Lysinibacillus* 1 isolate 1 genotype (1,0,0)†
Actinobacteria 1 isolate 1 genotype	*Actinobacteria*	*Actinobacteridae*	*Micrococcaceae*	*Micrococcus* 1 isolate 1 genotype (0,1,0)
Bacteroidetes 15 isolates 14 genotypes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Aquimarina* 12 isolates 11 genotypes (4,7,0) *Tenacibaculum* 2 isolates 2 genotypes (0,2,0) *Kordia* 1 isolate 1 genotype (0,1,0)
Proteobacteria 262 isolates 139 genotypes	*Gamma-* 82 isolates 57 genotypes	*Vibrionales* 39 isolates 33 genotypes	*Vibrionaceae*	*Vibrio* 35 isolates 31 genotypes (8,23,0) *Photobacterium* 4 isolates 2 genotypes (0,2,0)
		Alteromonadales 37 isolates 19 genotypes	*Alteromonadaceae*	*Microbulbifer* 17 isolates 4 genotypes (0,3,1)
			Shewanellaceae	*Shewanella* 14 isolates 10 genotypes (8,2,0)
			Pseudoalteromonadaceae	*Pseudoalteromonas* 5 isolates 4 genotypes (0,4,0)
			Colwelliaceae	*Colwellia* 1 isolate 1 genotype (0,1,0)
		Oceanospirillales 6 isolates 5 genotypes	*Oceanospirillaceae*	*Amphritea* 2 isolates 2 genotypes (1,1,0)
			Hahellaceae	*Endozoicomonas* 2 isolates 1 genotype (0,1,0) Unclassified 2 isolates 2 genotypes (0,2,0)
	Alpha- 180 isolates 82 genotypes	*Rhodobacterales*	*Rhodobacteraceae*	*Pseudovibrio* 125 isolates 49 genotypes (9,37,3) *Ruegeria* 49 isolates 27 genotypes (9,15,3) *Roseovarius* 1 isolate 1 genotype (0,1,0) *Phaeobacter* 1 isolate 1 genotype (1,0,0) Unclassified 4 isolates 4 genotypes (1,3,0)
Total				279 isolates 155 genotypes (42,106,7)

*Genus-level classification based on 16S rRNA gene sequencing.

†A genotype is defined as one unique BOX-PCR fingerprint. In brackets: number of genotypes found exclusively in *I. variabilis*, exclusively in *S. spinosulus* and in both species (shared genotypes), respectively.

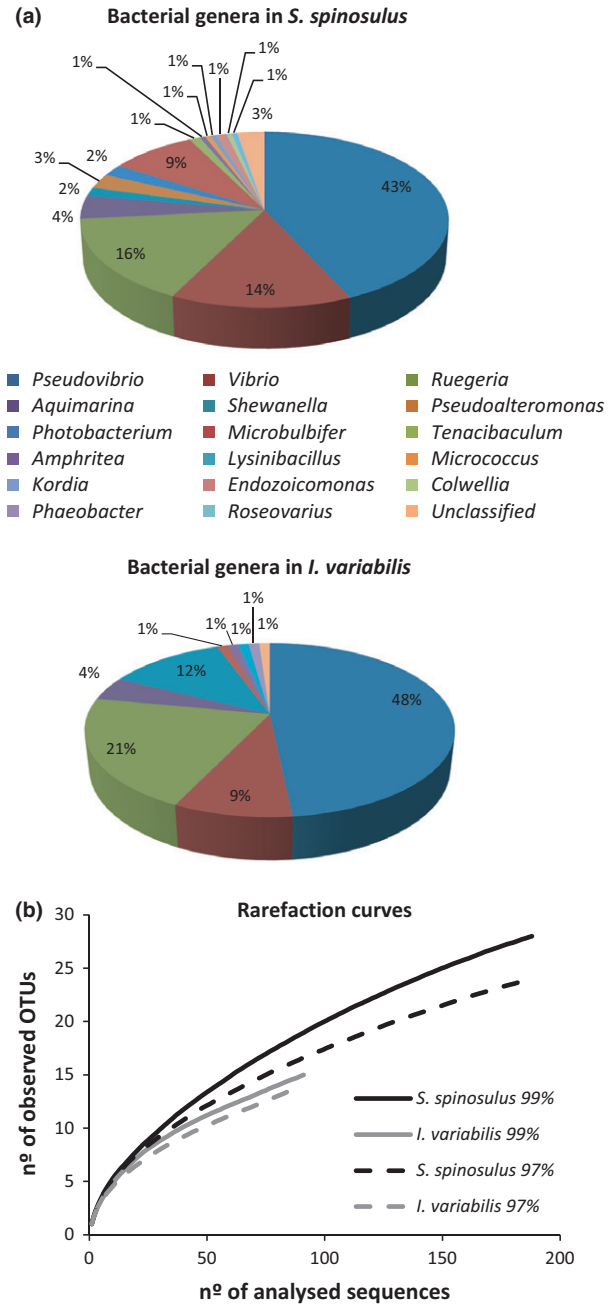

Fig. 1. Genus-level composition and 16S rRNA gene-based richness of bacteria isolated from *Sarcotragus spinosulus* and *Ircinia variabilis* (a) and rarefaction curves for both sequence libraries based on the number of observed 16S rRNA gene operational taxonomic units (OTUs) established at 97% and 99% gene similarity (b).

bilis (*c.* 54% of the isolates representing a distinct genotype). These results were particularly intriguing for the genera *Pseudovibrio*, *Ruegeria* and *Vibrio* that seemed to consist of genome-wide diverse taxa (Table 1, Fig. 2a-c) with a conserved 16S rRNA gene (Table 2, Figs 3 and 4). Strikingly, from the 155 genomes categorized by BOX-PCR

Table 2. Genotypic characterization of isolates

	*n**	OTUs 97†	OTUs 99‡	Chao1§	BOX¶	H'**
I. variabilis						
Pseudovibrio	44	1	1	1	12	1.004
Vibrio	8	2	3	4	8	0.903
Ruegeria	19	1	2	1	12	0.958
All isolates	91	14	15	24	49	1.560
S. spinosulus, normalized						
Pseudovibrio	42	1	1	1	19	1.043
Vibrio	17	2	3	2	15	1.160
Ruegeria	11	1	1	1	8	0.822
All isolates	91	14	17	24	60	1.629
S. spinosulus, full						
Pseudovibrio	81	1	1	1	40	1.305
Vibrio	27	3	4	3	23	1.342
Ruegeria	30	1	1	1	18	1.139
All isolates	188	24	28	37	113	1.886
I. variabilis + *S. spinosulus*						
Pseudovibrio	125	1	1	1	49	1.396
Vibrio	35	4	5	4	31	1.475
Ruegeria	49	1	2	1	27	1.240
All isolates	279	29	36	38	155	1.991

*Number of isolates.
†Number of 16S rRNA gene operational taxonomic units established at 97% sequence similarity.
‡Number of 16S rRNA gene operational taxonomic units established at 99% sequence similarity.
§Chao1 richness estimate calculated from OTU data, established at 97% 16S rRNA gene sequence similarity.
¶Number of BOX-PCR genotypes.
**Shannon diversity index of BOX-PCR profiles.

fingerprinting, only seven were shared between *I. variabilis* and *S. spinosulus*, with 42 and 106 genotypes being exclusive to each species, respectively. Six of the seven shared BOX-PCR genotypes belonged to the dominant genera *Pseudovibrio* and *Ruegeria*, while the 7th genotype common to both species represented one *Microbulbifer* strain. Interestingly, not a single *Vibrio* genotype was common to both sponge hosts (Table 1).

Phylogenetic inference

The genus *Pseudovibrio* clearly dominated the culturable bacterial communities of both sponge species under the conditions used in this study (Fig. 1). Except for one specimen of *S. spinosulus*, these isolates were prevalent in all surveyed sponge specimens. Closest matches to our *Pseudovibrio* isolates included bacteria previously found in bryozoans from the Adriatic Sea (*Pseudovibrio* sp. B411, Heindl *et al.*, 2010) and from other sponge species such as *Halichondria panicea* from the North Sea ('Sponge bacterium isolate3', Wichels *et al.*, 2006) and *Axinella dissimilis* from Irish waters (*Pseudovibrio* sp. Ad32, O'Halloran *et al.*, 2011) (Fig. 3). Phylogenetic analysis confirmed the high 16S rRNA gene conservation within the identified *Pseudovibrio* isolates (Fig. 3), in accordance with OTU richness

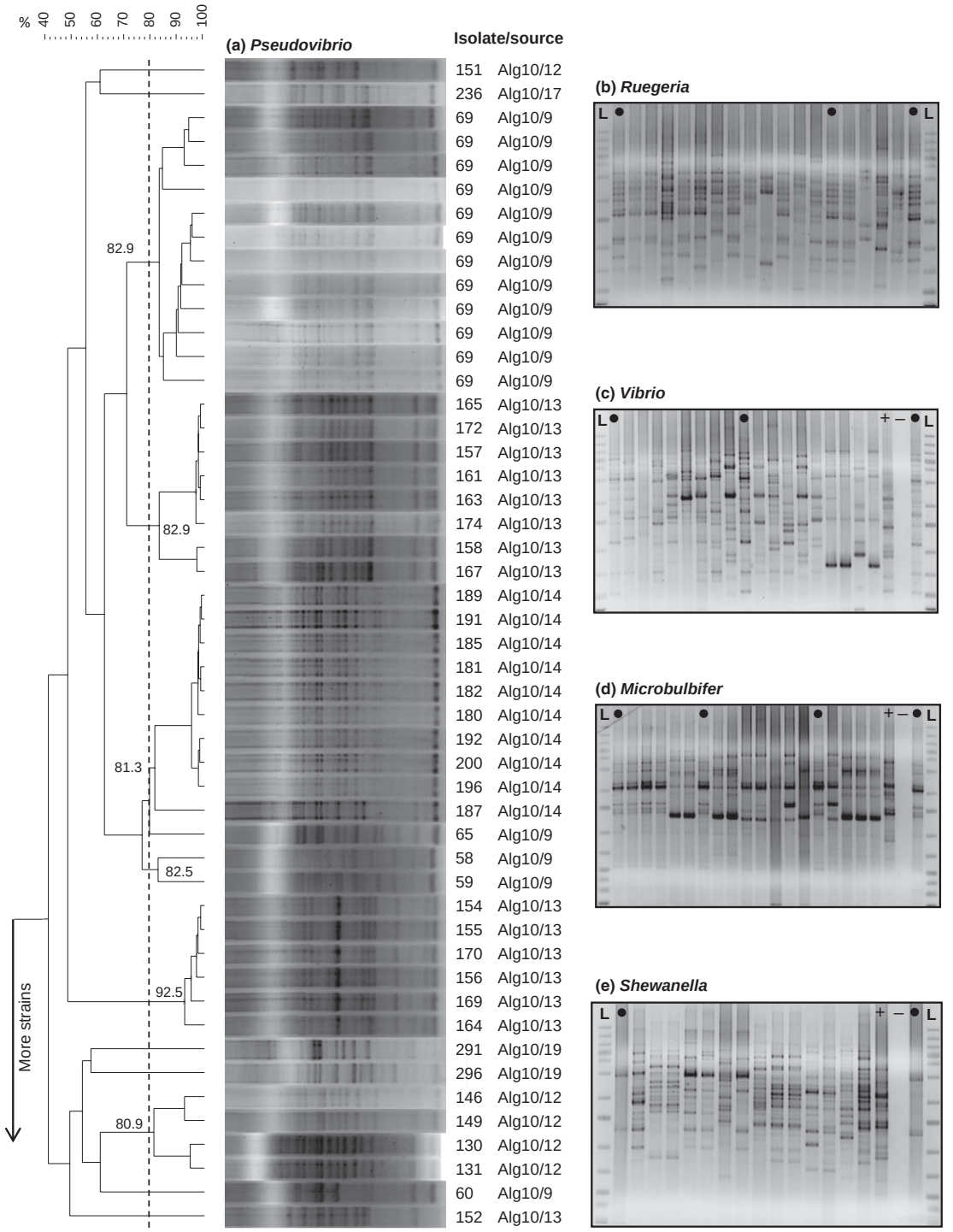

Fig. 2. BOX-PCR fingerprints. Left panel: Pearson correlation–UPGMA cluster analysis of *Pseudovibrio* BOX-PCR profiles (a). The group similarity retrieved for independent PCR replicates of the same isolate loaded across different gels (e.g. isolate 69) is used as a reference cut-off (dashed line) for the determination of genotype richness within each bacterial genus. Next to isolate codes are the sponge specimens from where each isolate has been retrieved. To note is the specimen-dependent occurrence of BOX-PCR genotypes. Alg10/12, Alg10/13, Alg10/14: *Ircinia variabilis* specimens. Alg 10/9, Alg 10/17, Alg10/19: *Sarcotragus spinosulus* specimens. The panel exemplarily shows 38 of the 125 analysed *Pseudovibrio* fingerprints. Right panel: examples of BOX-PCR fingerprinting raw data retrieved for *Ruegeria* (b), *Vibrio* (c), *Microbulbifer* (d) and *Shewanella* (e). Solid circles mark fingerprints corresponding to replicate PCRs of the same isolate. L, 1-kb DNA Ladder. On *Vibrio*, *Microbulbifer* and *Shewanella* panels: +, positive control; –, negative control.

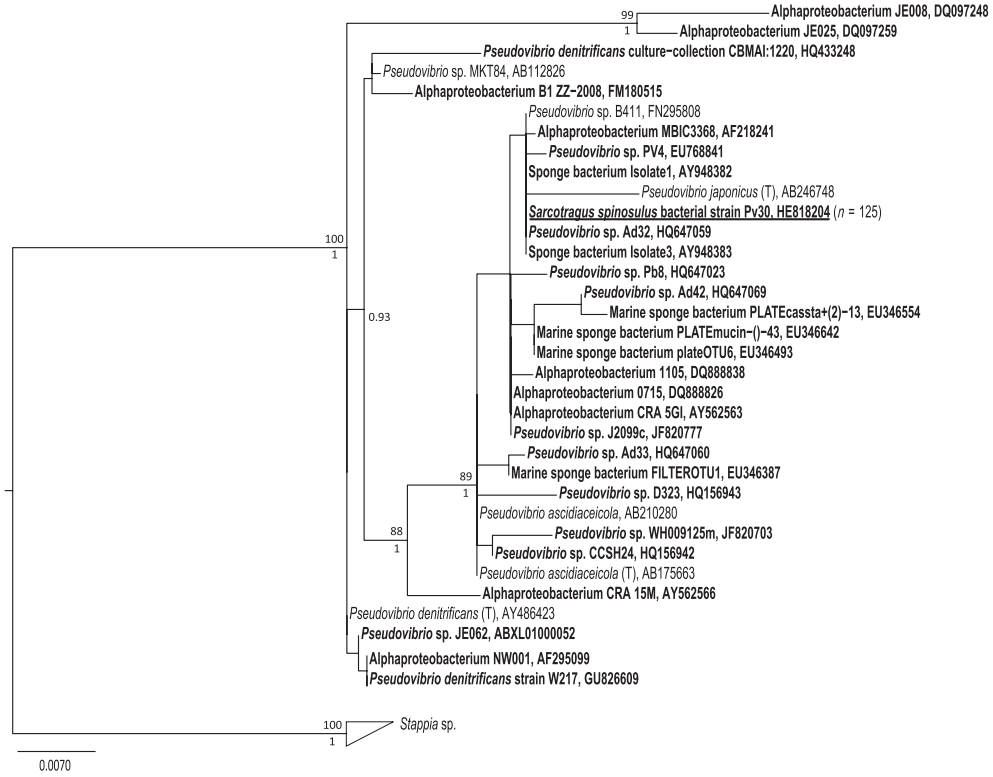

Fig. 3. Optimal 16S rRNA gene maximum-likelihood (ML) tree for *Pseudovibrio* spp. Isolates retrieved in this study and sponge-associated bacteria are highlighted in bold, and the former are underlined. In brackets is the number of isolates from this study, if more than one that could be represented by one single tree leaf. ML bootstrap values (> 70%) and bayesian posterior probabilities (> 0.95) are shown above and below branches, respectively. The tree is rooted with the genus *Stappia* (*Alphaproteobacteria*, *Rhodobacteraceae*).

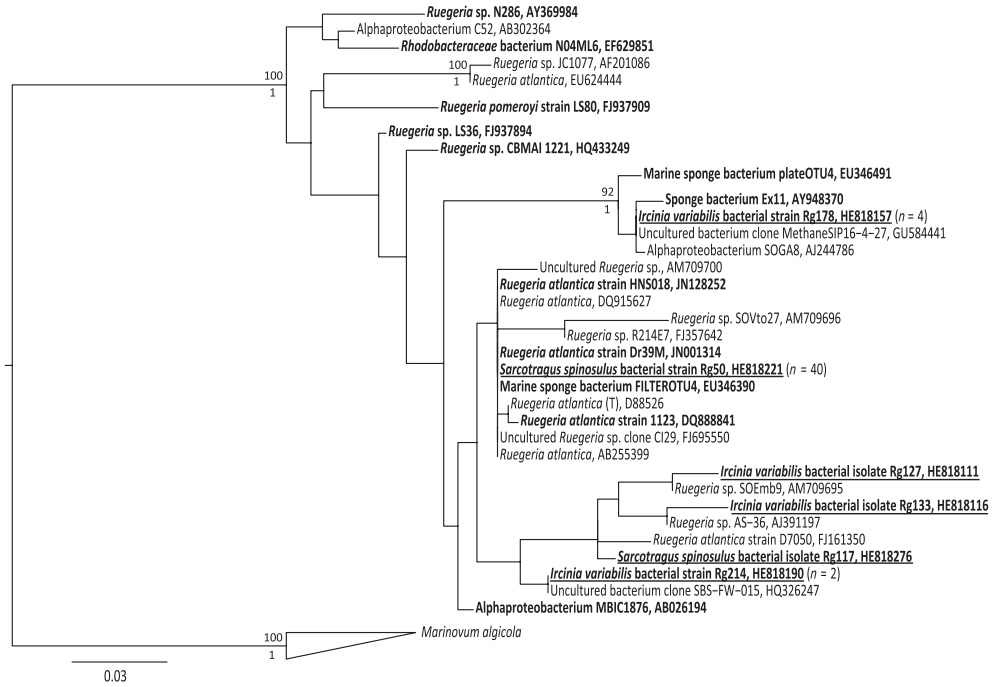

Fig. 4. Optimal maximum-likelihood (ML) tree for *Ruegeria* spp. Details are as provided for Fig. 2. The tree is rooted with the species *Marinovum algicola* (*Alphaproteobacteria*, *Rhodobacteraceae*).

estimates and in spite of their high genotypic variability as determined by whole-genome fingerprinting (Fig. 2a, Table 2). *Pseudovibrio* isolates from the inspected irciniid sponges were more closely related to the species *P. ascidiaceicola* and *P. japonicus* ($\geq$ 99.7% gene similarity) than to the species *P. denitrificans* (gene similarity = 98.7%) (Fig. 3).

Ruegeria isolates were also highly conserved at the 16S rRNA gene level, with only one OTU identified at a 97% gene similarity cut-off (Table 2). It was, nevertheless, possible to subdivide the two OTUs determined at 99% cut-off into a further six types by phylogenetic analysis (Fig. 4), and 27 different genotypes could be identified using BOX-PCR (Table 2), some of which are exemplarily shown (Fig. 2b). Closest matches to our *Ruegeria* isolates included bacteria isolated from sediments of marine hydrocarbon seeps ('Uncultured bacterium clone MethaneSIP16-4-27', Redmond *et al.*, 2010), corals collected in the Indian Ocean ('Uncultured *Ruegeria* sp. clone CI29', Nithyanand *et al.*, 2011) and other sponge species such as *Haliclona* sp. from the North Pacific Ocean ('Marine sponge bacterium FILTEROTU4', Sipkema *et al.*, 2011) (Fig. 4). The resemblance of *Ruegeria* isolates uncovered in this study to strains of the species *Ruegeria atlantica*, some of which isolated from marine sponges (Fig. 4), lied at 98.5–100.0% similarity at the primary 16S rRNA gene sequence level. *Ruegeria atlantica* (T) D88526 indeed represented, from among the type strains of the genus *Ruegeria*, the closest 16S rRNA gene relative to our *Ruegeria* sequences.

Vibrio isolates (n = 35) could be categorized in four distinct OTUs (97% cut-off, Table 2), clearly recognizable by phylogenetic inference (data not shown). The most dominant *Vibrio* OTU encompassed 29 isolates. Isolates within this OTU often showed about 99.8% 16S rRNA gene homology to type strains of the species *V. gigantis* and *V. crassostreae* and, surprisingly, 100% homology (800 bp) to *Vibrio* sp. H455 isolated from the marine sponge *Bubaris* sp. in the West Atlantic Ocean (Hoffmann *et al.*, 2010). The other three OTUs resembled type strains of the species *V. gallaecicus* (n = 2, 96.4% gene homology), *V. breoganii* (n = 3, 99.3% gene homology) and *V. atypicus* (n = 1, 97.4% gene homology). Noticeably, 31 distinct BOX-PCR genotypes were detected within our 35 *Vibrio* isolates (see Fig. 2c).

Three putative new species with NCBI sequence matches below 97% to previously reported bacteria were found in this study (Table 3). They affiliated with the family *Hahellaceae* (isolates Ez249 and Ez302, Fig. 5) and to an unclassified bacterial lineage in the family *Rhodobacteraceae* (isolate Ph113, Fig. 6). Isolates Ez249 and Ez302 were phylogenetically close but do not belong to the recently described genus *Endozoicomonas* (Fig. 5).

Table 3. Putative new bacterial species

Isolates	Taxonomic affiliation*	Closest 16S rRNA gene relative/ Isolation source†	%‡	Closest type strain§	%
Ap52, Ap210	*Amphritea* (100%)	*Amphritea* sp. MEBiC05461T (GU289646) – Marine sponge	98	*Amphritea atlantica* (T) M41 (AM156910)	96
Aq132, Aq135, Aq141, Aq142	*Aquimarina* (100%)	*Aquimarina macrocephali* (T) JAMB N27 (AB517144) – Sediment	98	*Aquimarina macrocephali* (T) JAMB N27 (AB517144)	98
Aq77, Aq345, Aq349	*Aquimarina* (100%)	*Flavobacterium* sp. S4487 (FJ457299) – Seaweed	98	*Aquimarina macrocephali* (T) JAMB N27 (AB517144)	97
Ez249	*Hahellaceae* (82%)	Intranuclear bacterium (FM244838) – *Bathymodiolus childressi* (mussel)	94	*Kistimonas asteriae* (T) KMD 001 (EU599216)	94
Ez289	*Endozoicomonas* (100%)	*Endozoicomonas elysicola* (T) MKT110 (AB196667) – Sea slug	98	*Endozoicomonas elysicola* (T) MKT110 (AB196667)	98
Ez302	*Hahellaceae* (85%)	Uncultured bacterium clone Past_L08 (GU119134) – *Porites astreoides* (coral)	95	*Endozoicomonas elysicola* (T) MKT110 (AB196667)	94
Ph82	*Rhodobacteraceae* (100%)	Marine sponge bacterium plateOTU12 (EU346499) - *Haliclona* sp.	99	*Leisingera aquimarina* (T) LMG 24366 (AM900415)	98
Ph113	*Rhodobacteraceae* (100%)	Uncultured bacterium clone RESET_18C 11 (JN874120) – Hydrothermal plume	98	*Shimia marina* (T) CL-TA03 (AY962292)	97
Ph212	*Rhodobacteraceae* (100%)	*Roseobacter* sp. 38.98 (AY870684) – Oyster	99	*Roseovarius crassostreae* (T) CV919-312 (AF114484)	96
Ph303	*Rhodobacteraceae* (100%)	*Phaeobacter* sp. UDC400 (HM031990) – Seawater	99	*Phaeobacter gallaeciensis* (T) BS107T (Y13244)	97

*RDP Classifier results are shown, using 80% confidence threshold as cut-off for taxonomic assignment. Confidence thresholds (%) are shown in brackets.

†Closest relatives were determined using the Basic Local Alignment Search Tool (BLAST) of The National Center for Biotechnology Information (NCBI).

‡Per cent homology between query sequences and closest matches.

§Closest type strains were searched using the sequence match tool of RDP.

Their closest phylogenetic relatives were both uncultured bacteria retrieved from other marine invertebrates at rather low levels of gene similarity (Table 3, Fig. 5). For the *Rhodobacteraceae* isolate Ph113, no sponge-derived bacterial sequences were found among its 100 closest matches present in public databases. It resembled uncultured bacterial clones and displayed some degree of phylogenetic relatedness to the genus *Phaeobacter* (Fig. 6). Using less stringent criteria, and thus acknowledging isolates sharing $\leq$ 98.5% 16S rRNA gene similarity to their closest cultured relatives as potentially novel taxa, a few more putatively new species could be found (Table 3). Noteworthy among them were phylotypes in the genus *Aquimarina* that formed well-delineated, sponge species-specific clusters (Fig. 7, Table 3). The four OTUs determined for this genus by rarefaction analysis were well discriminated by phylogenetic inference and are represented by 3 clusters (I-III) comprising 11 isolates in addition to one further single sequence entry (Aq107) in the *Aquimarina* tree (Fig. 7). Finally, although several other phylotypes in the *Rhodobacteraceae* family (e.g. strains Ph82, Ph212 and Ph303) displayed high similarity to previously cultured bacteria, they appeared to belong to lineages for which type species have not yet been described (Table 3).

NRPS and PKS genes

PKS- and NRPS-specific PCR was performed for each BOX-PCR representative. The presence of PKS/NRPS genes was considered positive whenever a strong unambiguous amplicon of the right size showing no side bands could be observed (Fig. 7). For PKS gene screening, *Pseudovibrio* (47 in 49) and *Aquimarina* (10 in 11) genotypes delivered such amplicons. High-quality sequences could be obtained for 26 and 8 genotypes in each genus, respectively. The retrieved PKS ketosynthase domain gene sequences were compared with those in the GenBank database using BLASTX (Table 4). In general, translated *Pseudovibrio* PKS gene sequences showed high similarity (97–100%) to previously described ketosynthase protein domains of *Pseudovibrio* strains isolated from other sponge species in Irish coastal waters (O'Halloran *et al.*, 2011) (Table 4). For *Aquimarina*, four of the sequences obtained were clonal and represented isolates from phylogenetic cluster I (Fig. 7). These sequences showed low homology (51–53%) to a type I polyketide synthase domain from *Mycobacterium* sp. (*Actinobacteria*, *Mycobacteriaceae*). Interestingly, translated PKS gene sequences from *Aquimarina* isolates in cluster III (Fig. 7) showed homologies with onnamide and bryostatin type I polyketide synthases from sponge and bryozoan symbionts, respectively (Table 4). Although *Aquimarina* strains from cluster II produced clean PKS PCR signals (Fig. 7), no sequences of satisfactory quality were obtained for these products. Screening for NRPS genes very often resulted in multiple unspecific PCR signals, and unambiguous amplicons of the expected size were not observed.

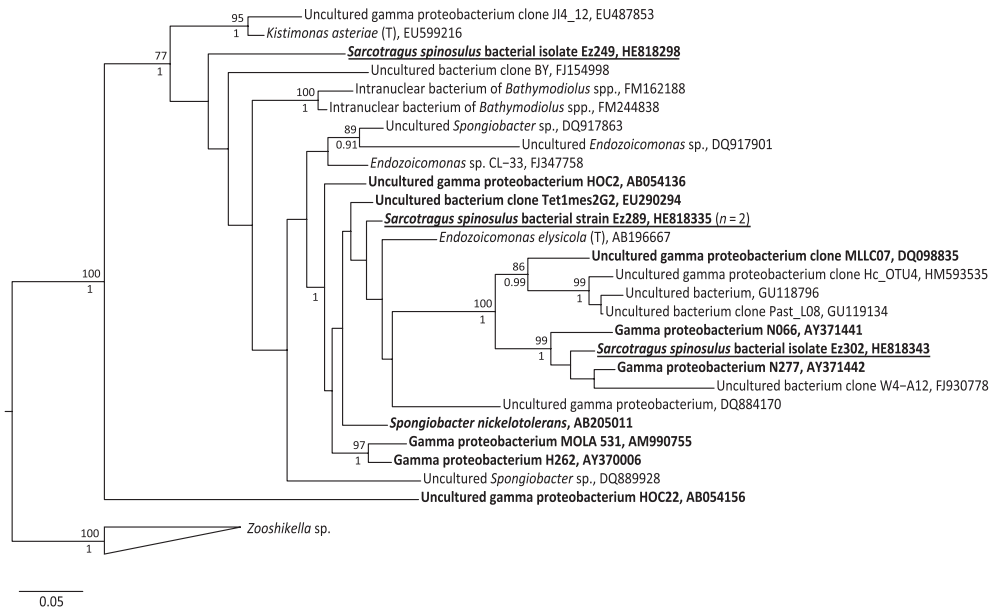

Fig. 5. Optimal 16S rRNA gene maximum-likelihood (ML) tree for relatives within the family *Hahellaceae*, including *Endozoicomonas* phylotypes. Details are as provided for Fig. 2. The tree is rooted with the genus *Zooshikella* (*Gammaproteobacteria*, *Hahellaceae*).

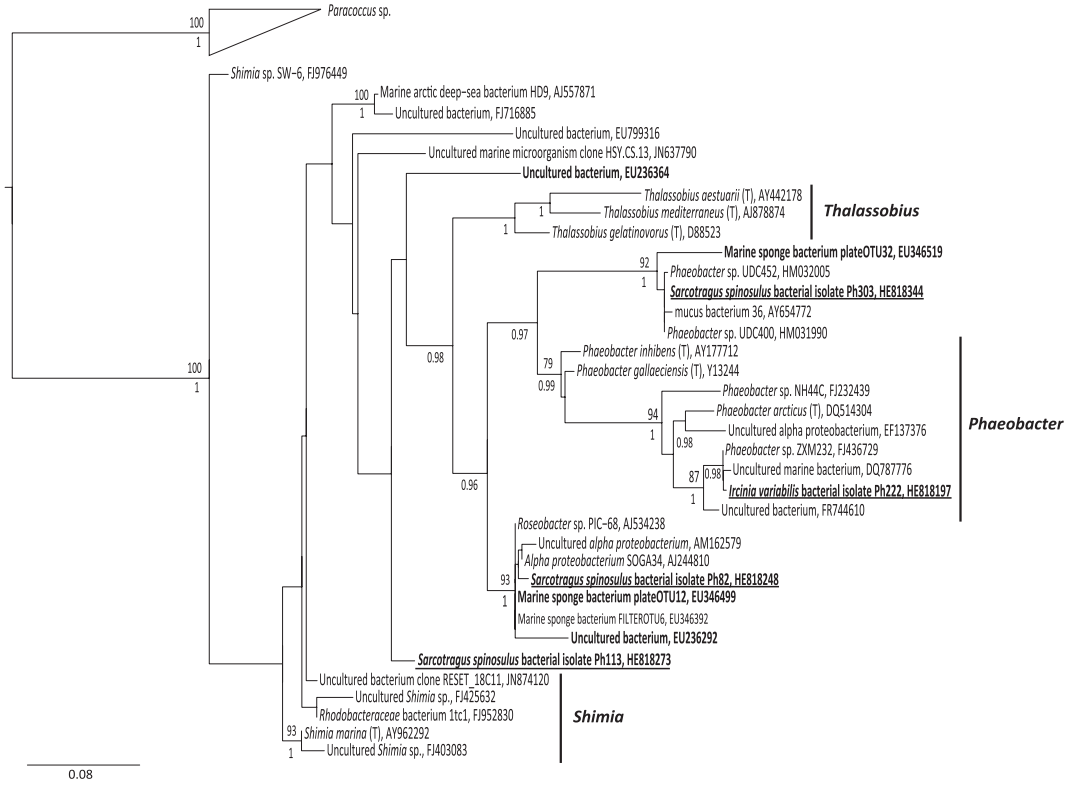

Fig. 6. Optimal 16S rRNA gene maximum-likelihood (ML) tree for relatives within the family *Rhodobacteraceae*, including members of the genus *Phaeobacter*. Details are as provided for Fig. 2. The tree is rooted with the genus *Paracoccus* (*Alphaproteobacteria*, *Rhodobacteraceae*).

Antagonism assays

The 155 different genotypes were screened for antimicrobial activity using a double-layer antagonism assay, with 53 (34%) demonstrating antimicrobial activity against at least one of the indicator strains tested. Eighteen (12%) isolates showed antimicrobial activity against both strains, 27 (17%) isolates were positive against *S. aureus* and 44 (28%) against *E. coli*. *Vibrio* isolates showed to be the most active, with 27 (84%) *Vibrio* isolates active against

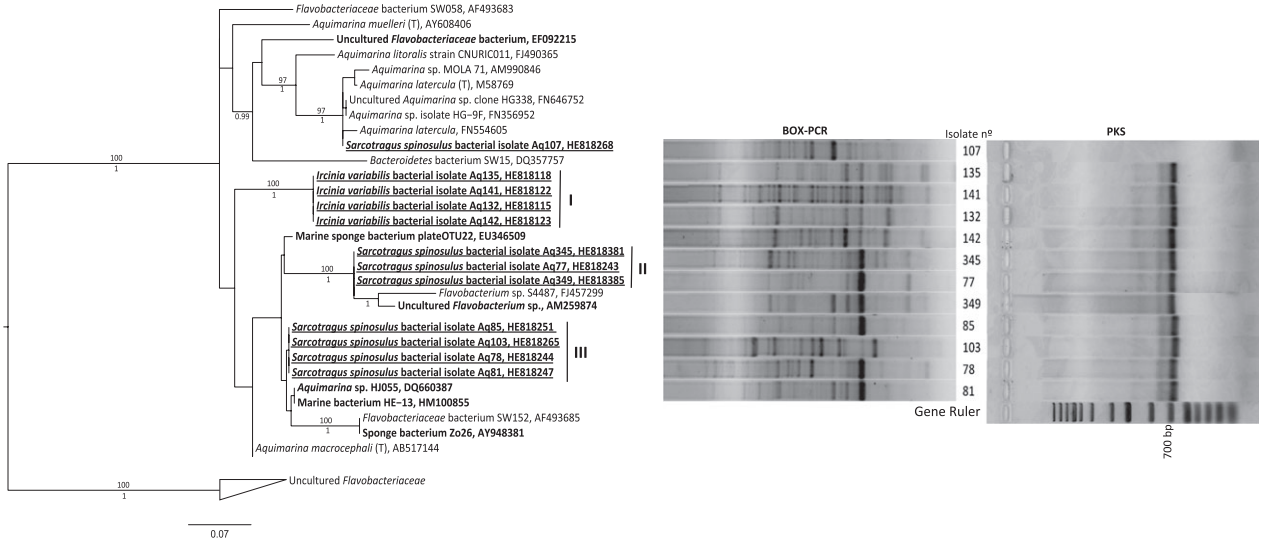

Fig. 7. Optimal 16S rRNA gene maximum-likelihood (ML) tree, BOX-PCR profiles and PKS gene screening for the genus *Aquimarina*. Tree details are as provided for Fig. 2. The tree is rooted with the family *Flavobacteriaceae* (*Bacteroidetes*).

Table 4. Closest matches of PKS sequences

Isolate*	Classification	Closest match (BLASTX) (Accession number – description)	Coverage (%)	Homology (%)
Pv227 (*n* = 8)	*Pseudovibrio*	ADY17934 – ketosynthase domain protein [*Pseudovibrio* sp. Ad28]	99	98–100
Pv119 (*n* = 12)	*Pseudovibrio*	ADY17930 – ketosynthase domain protein [*Pseudovibrio* sp. Ad23]	99	97–100
Pv69 (*n* = 3)	*Pseudovibrio*	ADY17935 – ketosynthase domain protein [*Pseudovibrio* sp. Ad30]	99–100	98–99
Pv35 (*n* = 1)	*Pseudovibrio*	ADY17940 – ketosynthase domain protein [*Pseudovibrio* sp. Ad48]	99	97–99
Pv61 (*n* = 1)	*Pseudovibrio*	ADY17929 – ketosynthase domain protein [*Pseudovibrio* sp. Ad17]	99	99
Pv97 (*n* = 1)	*Pseudovibrio*	YP_006269102 – polyketide synthase [*Actinoplanes* sp. SE50/110]	92	65
Aq142 (*n* = 4)	*Aquimarina*	ACZ54286 – type I polyketide synthase [*Mycobacterium* sp. CNJ-823 PL04]	97–99	51–53
Aq78 (*n* = 1)	*Aquimarina*	ADD65274 – type I ketosynthase [uncultured bacterium]	98	56
		AAR19304 – putative type I polyketide synthase [symbiont bacterium of *Paederus fuscipes*]	99	46
Aq81 (*n* = 3)	*Aquimarina*	ADF57409 – polyketide synthase [uncultured bacterium]	98	62
		AAV97870 – OnnB [symbiont bacterium of *Theonella swinhoei*]	99	53
		ABM63528 – BryC [Candidatus *Endobugula sertula*]	99	53
		AAR19304 – putative type I polyketide synthase [symbiont bacterium of *Paederus fuscipes*]	99	52

*In brackets is the number of BOX-PCR genotypes sharing a closest PKS relative.

E. coli and 12 (38%) active against *S. aureus*. Sixty per cent of *Shewanella* isolates were positive against both strains, whereas 58% of *Aquimarina* isolates were active against *S. aureus*. *Ruegeria* and *Pseudovibrio* were the least active isolates with only one *Ruegeria* isolate showing mild antimicrobial activity against *S. aureus* and two *Pseudovibrio* isolates active against *E. coli*. Table 5 summarizes *in vitro* antagonistic activity results for the dominant bacterial genera found in this study.

Table 5. Antimicrobial activity per bacterial genus

	Antimicrobial activity	
Genus (BOX-PCR genotypes)	*S. aureus* (%)	*E. coli* (%)
Pseudovibrio (*n* = 49)	0	4
Ruegeria (*n* = 27)	4	0
Vibrio (*n* = 31)	38	84
Microbulbifer (*n* = 4)	0	50
Shewanella (*n* = 10)	60	80
Aquimarina (*n* = 11)	58	8
Others (*n* = 23)	4	15
Total (*n* = 155)	17	28

Discussion

The culturable bacterial community of Irciniidae sponges consisted of a common core composed of *Pseudovibrio*, *Ruegeria* and *Vibrio*, which were notably recovered from most analysed specimens and several other less abundant and primarily proteobacterial genera. Equivalent genotypic richness and diversity measures were observed for both species, despite the trend for slightly higher values in *S. spinosulus*. Previous results using a cultivation-independent technique (PCR-DGGE) supported the picture of moderately higher bacterial richness and diversity in *S. spinosulus* than in *I. variabilis* (Hardoim *et al.*, 2012). Other cultivation-dependent studies documented similar genus-level composition for sponge species from different locations under varied culture conditions, particularly regarding the dominance of *Alphaproteobacteria* such as *Pseudovibrio* and *Ruegeria* (Webster & Hill, 2001; Thiel & Imhoff, 2003; Lafi *et al.*, 2005; Muscholl-Silberhorn *et al.*, 2008; Sipkema *et al.*, 2011). Notably, *Pseudovibrio* spp. may even dominate in sponge larvae as demonstrated for the species *P. denitrificans* (Enticknap *et al.*, 2006), suggesting it may be a symbiont of potential value in the interaction with its hosts. Bacteria from this genus have also been isolated from other marine sources such as seawater, ascidians, tunicates and corals (O'Halloran *et al.*, 2011; and ref. therein). Thus, they seem to be a group of versatile microorganisms (Shieh *et al.*, 2004) with adaptive traits for both symbiotic and free-living life strategies, as evidenced by the first comparative analysis of sequenced *Pseudovibrio* genomes (Bondarev *et al.*, 2011). The genus *Ruegeria*, in its turn, belongs – along with *Phaeobacter* – to the ecologically relevant *Roseobacter* clade, which contains several widespread, abundant, physiologically versatile and primarily marine bacterial genera that form a well-supported clade in the family *Rhodobacteraceae* (Brinkhoff *et al.*, 2008). Most *Ruegeria* isolates from this study are closely related to *Ruegeria atlantica* (Fig. 4), originally isolated from north-eastern Atlantic Ocean bottom sediments and subsequently found in seawater and marine macroorganisms, including other sponge species (Muscholl-Silberhorn *et al.*, 2008; Menezes *et al.*, 2010). *Vibrio* was the most profuse *Gammaproteobacteria* genus found in this work. Several cultivation-dependent and cultivation-independent studies have shown that vibrios populate in and/or on marine organisms, such as corals, fish, molluscs, seagrasses, sponges, shrimps and zooplankton (Thompson *et al.*, 2004; and refs. therein).

Eventual increments in the abundance of *Vibrio* spp. in these settings, often caused by rising seawater temperatures, may be related with their acknowledged pathogenicity, resulting in increased oyster mortality in summer (Garnier *et al.*, 2007) and coral bleaching (Rosenberg *et al.*, 2007). The high frequency of *Vibrio* in marine sponges suggests these invertebrates as vectors of likely commensal, but potentially pathogenic bacteria that may cause infection and disease in sponges or other marine macroorganisms (Alves *et al.*, 2010). In this regard, a recent survey alerts for the likely participation of *Vibrio rotiferianus* in a disease outbreak affecting *Ircinia variabilis* specimens in the Mediterranean Sea (Stabili *et al.*, 2012). With 35 isolates and 31 different genotypes, *Vibrio* is the most diversified bacterial genus in this study, supporting the raised notion of sponges as sources of taxonomic and phylogenetic variability within this bacterial taxon (Hoffmann *et al.*, 2010).

Ten bacterial phylotypes retrieved in this study were regarded as potential candidates for new species (Table 3). Most conspicuously, isolate Ez249 showed 94% homology with an intranuclear bacterium found in the mussel *Bathymodiolus* sp. and clustered with other unclassified bacteria showing some degree of resemblance to the genus *Endozoicomonas*. This phylogenetic affiliation tempts us to speculate that Ez249 could be a sponge-associated bacterium living in the nucleus of sponge cells. Although apparently little effort has been made to address the phylogeny of possible intranuclear sponge symbionts, their existence is supported by early (Vacelet, 1970) and recent (Maldonado *et al.*, 2012) electron microscopy documentations. Similarly, isolate Ez302 affiliates with an otherwise exclusively uncultured bacterial clade also closely related to *Endozoicomonas* (Fig. 5). The genus *Endozoicomonas* was recently established with the discovery of the type strain *Endozoicomonas elysicola*, isolated from a sea slug (Kurahashi & Yokota, 2007), and remains a poorly resolved genus, with neither a clearly attributed function nor complete genome sequences available. Overall, the finding of potential new species using a rather widespread isolation procedure suggests marine sponges are keystone reservoirs of bacterial phylogenetic novelty. This observation is strengthened by the plethora of molecular-based studies of the sponge-associated microbiome, in which several sponge-specific bacterial phylogenetic signatures have been consistently found (Taylor *et al.*, 2007; Simister *et al.*, 2012). However, sponge-associated bacterial phylotypes retrieved in cultivation-independent studies often do not match the phylogeny of the corresponding culturable microbiota. Indeed, dominant symbionts found previously in *I. variabilis* and *S. spinosulus* by cultivation-independent means belonged to uncultured lineages in the phyla *Proteobacteria*, *Actinobacteria* and *Chloroflexi* (Hardoim *et al.*, 2012), which showed no phylogenetic overlap with the bacterial cultures described in this study. This stresses the need of employing alternative culture strategies in future studies to capture further sponge-derived bacterial diversity in the laboratory, improving thereby our access to the metabolic features of symbiotic microorganisms in the marine realm (Sipkema *et al.*, 2011).

While 16S rRNA gene sequencing provides information on the phylogenetic position of unknown isolates, it often does not allow discrimination between strains of the same species or even different species, which usually share highly conserved 16S rRNA genes (Rodríguez-Valera, 2002). Our analysis clearly demonstrates that 16S rRNA gene phylogeny does not recapitulate genome diversity at the strain level. Using randomly amplified polymorphic DNA (RAPD) analysis, O'Halloran *et al.* (2011) found likewise 33 genotypes within 73 sponge-derived *Pseudovibrio* isolates. Genomic rearrangements resulting from the traffic of mobile genetic elements (MGEs) such as plasmids, phages and transposons within and between genomes are overarching factors fostering bacterial genome evolution and diversification that escape detection by regular house-keeping gene analyses, but that can be diagnosed by molecular genotyping (Ishii & Sadowsky, 2009). Such rearrangements might often underpin subtle to sharp differentiation of ecological value – for instance, the acquisition of a pathogenicity or antibiotic resistance island (Van Elsas *et al.*, 2011) – between otherwise clonal strains. Events of gene loss and acquisition are known to override genome evolution within the *Proteobacteria* (Costa *et al.*, 2009; Touchon *et al.*, 2009; Van Elsas *et al.*, 2011), one of the prevailing sponge-associated bacterial phyla. Recent sponge metagenomic studies revealed high incidence of MGEs in these settings and highlight marine sponges as microniches conducive to horizontal gene transfer (HGT) within sponge symbionts (Thomas *et al.*, 2010; Fan *et al.*, 2012). The conspicuous genotypic diversification observed in our analyses for isolates sharing high levels of 16S rRNA gene homology is suggestive of a host specimen-dependent composition, at the strain level, of prevalent bacteria in marine sponges. This could result from independent genomic rearrangement events experienced by closely related symbionts thriving in different host specimens.

In the present study, high-quality PKS gene sequences were obtained for *Pseudovibrio* and *Aquimarina* isolates. The *Pseudovibrio* ketosynthase (KS) domain sequences from this study showed high homology to *Pseudovibrio* spp. KS sequences from *Polymastia boletiformis*, *Axinella dissimilis* and *Haliclona simulans* sponge species collected in the coast of Ireland (O'Halloran *et al.*, 2011). The presence of PKS and NRPS genes in the *Pseudovibrio* genus has been previously reported in whole-genome annotations (Bondarev *et al.*, 2011). Further PKS sequences retrieved for some *Aquimarina* isolates (Fig. 7, Table 5) represent

novel sequences with low to moderate homology to PKS sequences in the NCBI database (Table 4). Noteworthy among them are those resembling PKSs involved in the biosynthesis of the symbiont-derived and antitumor compounds onnamide (Piel *et al.*, 2004) and bryostatin (Sudek *et al.*, 2007). To our knowledge, this is the first report on PKS genes in *Aquimarina* species. The hybrid polyketide/nonribosomal peptides ariakemicins A and B from *Rapidithrix* sp. HC35 (Oku *et al.*, 2008) and the polyketide elansolide D from *Chitinophaga pinensis* DSM 2588 (Teta *et al.*, 2010) are among the first compounds of polyketide nature reported for the phylum *Bacteroidetes*, whose genomes appear to present low densities of PKS- and NRPS-encoding genes (Donadio *et al.*, 2007).

Regarding *in vitro* antimicrobial activity, we registered 53 different genotypes (34%) inhibiting the growth of at least one of the test strains. The antimicrobial activities reported in previous works regarding sponge-derived bacteria are very variable, ranging from 7.6% (Santos *et al.*, 2010) to 50% (Kennedy *et al.*, 2009). In our study, *Alphaproteobacteria* were the least active organisms, with only one *Ruegeria* isolate showing mild antimicrobial activity against *S. aureus* and 2 *Pseudovibrio* isolates active against *E. coli*. Conversely, previous studies reported on highly active *Alphaproteobacteria* (Hentschel *et al.*, 2001; O'Halloran *et al.*, 2011), particularly within the genus *Pseudovibrio* (Kennedy *et al.*, 2009; Santos *et al.*, 2010; O'Halloran *et al.*, 2011). However, such an antimicrobial activity in *Pseudovibrio* may be weak and unstable and easily lost during cultivation (Muscholl-Silberhorn *et al.*, 2008). In contrast, *Vibrio* spp. showed to be the most active bacterial genus, with remarkable antagonistic activity against *E. coli* (84%) and *S. aureus* (38%). Despite their ability to interact with eukaryotes, *Vibrio* spp. are underexplored for their capacity to produce bioactive secondary metabolites, with studies limited to only a few species (Mansson *et al.*, 2011). While their involvement in communication processes is already being addressed (Zan *et al.*, 2011), their antimicrobial properties and possible roles in sponge protection deserve further development. Although there is a tendency of directly linking the presence of PKS and NRPS genes to bioactivity (Zhang *et al.*, 2009a,b), these two features do not always overlap completely (Kennedy *et al.*, 2009; Schneemann *et al.*, 2010). In fact, the antimicrobial activity observed in this study, particularly within the *Vibrio* genus, was generally not related to polyketide synthase or nonribosomal peptide synthetase gene detection.

Given < 1% of sponge-associated bacteria is believed to be readily isolated using currently available cultivation techniques (Friedrich *et al.*, 2001), the bacterial diversity observed in the present study might in fact represent only a small fraction of the total community associated with Irciniidae sponges. Still, a simple and widespread cultivation methodology allowed us to retrieve considerable bacterial diversity and bioactivity, including potentially novel PKS genes found in the genus *Aquimarina*, several putative new bacterial species or strains within species and the non-PKS/NRPS-related antimicrobial activity detected in *Vibrio* isolates. These findings highlight the value of the microbiota cultured here as a promising source in future genome and metabolome mining for new bioactive molecules. The high genotypic variation observed within phylogenetically close strains prevailing in different sponge specimens poses further challenges to our understanding of marine sponge microbiome dynamics and the conservation of their functioning. In this context, dedicated approaches coupling alternative cultivation strategies to metagenomics and single-cell genomic methods will most likely not only bring about innovative solutions to the discovery of genes, enzymes and natural products from Irciniidae sponges, but also broaden the knowledge – and enable the linkage – of the functional and structural biodiversity spectrum within these holobionts.

Acknowledgements

We thank Prof. Margarida Reis (Centre for Environmental and Marine Sciences, University of Algarve) for gently conceding the bacterial strains *Staphylococcus aureus* NCTC 6571 and *Escherichia coli* NCTC 9001. The authors would like to acknowledge the Portuguese Foundation for Science and Technology (FCT) for financial support through the project PTDC/MAR/101431/2008. C.C.P. Hardoim has a Ph.D. scholarship granted by FCT (grant no. SFRH/BD/60873/2009). J.R. Xavier's research is funded by a FCT postdoctoral fellowship (grant no. SFRH/BPD/62946/2009). The authors have no conflict of interest to declare.

References

Alves N Jr, Neto OSM, Silva BSO *et al.* (2010) Diversity and pathogenic potential of vibrios isolated from Abrolhos Bank corals. *Environ Microbiol Rep* **2**: 90–95.

Ashelford KE, Chuzhanova NA, Fry JC, Jones AJ & Weightman AJ (2005) At Least 1 in 20 16S rRNA sequence records currently held in public repositories is estimated to contain substantial anomalies. *Appl Environ Microbiol* **71**: 7724–7736.

Bayer K, Scheuermayer M, Fieseler L & Hentschel U (2013) Genomic mining for novel $FADH_2$-dependent halogenases in marine sponge-associated microbial consortia. *Mar Biotechnol* **15**: 63–72.

Blunt JW, Copp BR, Keyzers RA, Munro MHG & Prinsep MR (2012) Marine natural products. *Nat Prod Rep* **29**: 144–222.

Bondarev V, Richter M, Piel J, Schwedt A & Schulz-Vogt HN (2011) The genus *Pseudovibrio* contains metabolically versatile and symbiotically interacting bacteria. In: Schwedt A (2011)

Physiology of a marine Beggiatoa strain and the accompanying organism Pseudovibrio sp. – a facultatively oligotrophic bacterium. PhD Thesis, Bremen University, Bremen.

Brinkhoff T, Giebel HA & Simon M (2008) Diversity, ecology, and genomics of the *Roseobacter* clade: a short overview. *Arch Microbiol* **189**: 531–539.

Cook SDC & Bergquist PR (2002) Family *Irciniidae* Gray, 1867. *Systema Porifera. A Guide to the Classification of Sponges* (Hooper JNA & Van Soest RWM, eds), pp. 1022–1027. Kluwer Academic/Plenum Publishers, New York, Boston, Dordrecht, London, Moscow.

Costa R, van Aarle I, Mendes R & van Elsas JD (2009) Genomics of pyrrolnitrin biosynthetic loci: evidence for conservation and whole-operon mobility within Gram-negative bacteria. *Environ Microbiol* **11**: 159–175.

Currie BJ, Gal D, Mayo M, Ward L, Godoy D, Spratt BG & LiPuma JJ (2007) Using BOX-PCR to exclude a clonal outbreak of melioidosis. *BMC Infect Dis* **7**: 68–74.

De Rosa S, Milone A, DeGiulio A, Crispino A & Iodice C (1996) Sulfated furanosesterterpenes from two sponges of the genus *Ircinia*. *Nat Prod Lett* **8**: 245–251.

De Rosa S, DeGiulio A, Crispino A, Iodice C & Tommonaro G (1997) Palinurin and fasciculatin sulfates from two Thyrrenean sponges of the genus *Ircinia*. *Nat Prod Lett* **10**: 7–12.

De Rosa S, Mitova M & Tommonaro G (2003) Marine bacteria associated with sponge as source of cyclic peptides. *Biomol Eng* **20**: 311–316.

Donadio S, Monciardini P & Sosio M (2007) Polyketide synthases and nonribosomal peptide synthases: the emerging view from bacterial genomics. *Nat Prod Rep* **24**: 1073–1109.

Enticknap JJ, Kelly M, Peraud O & Hill RT (2006) Characterization of a culturable alphaproteobacterial symbiont common to many marine sponges and evidence for vertical transmission via sponge larvae. *Appl Environ Microbiol* **72**: 3724–3732.

Fan L, Reynolds D, Liu M *et al.* (2012) Functional equivalence and evolutionary convergence in complex communities of microbial sponge symbionts. *PNAS* **109**: E1878–E1887.

Finking R & Marahiel MA (2004) Biosynthesis of nonribosomal peptides. *Annu Rev Microbiol* **58**: 453–488.

Fisch KM, Gurgui C, Heycke N *et al.* (2009) Polyketide assembly lines of uncultivated sponge symbionts from structure-based gene targeting. *Nat Chem Biol* **5**: 494–501.

Friedrich AB, Fischer I, Proksch P, Hacker J & Hentschel U (2001) Temporal variation of the microbial community associated with the mediterranean sponge *Aplysina aerophoba*. *FEMS Microbiol Ecol* **38**: 105–115.

Garnier M, Labreuche Y, Garcia C, Robert M & Nicolas JL (2007) Evidence for the involvement of pathogenic bacteria in summer mortalities of the pacific oyster *Crassostrea gigas*. *Microb Ecol* **53**: 187–196.

Giovannoni S & Stingl U (2007) The importance of culturing bacterioplankton in the 'omics' age. *Nat Rev Microbiol* **5**: 820–826.

Hardoim CCP, Esteves AIS, Pires FR, Gonçalves JMS, Cox C, Xavier JR & Costa R (2012) Phylogenetically and spatially close marine sponges harbour divergent bacterial communities. *PLoS ONE* **7**: e53029.

Heindl H, Wiese J, Thiel V & Imhoff JF (2010) Phylogenetic diversity and antimicrobial activities of bryozoan-associated bacteria isolated from Mediterranean and Baltic Sea habitats. *Syst Appl Microbiol* **33**: 94–104.

Hentschel U, Schmid M, Wagner M, Fieseler L, Gernert C & Hacker J (2001) Isolation and phylogenetic analysis of bacteria with antimicrobial activities from the Mediterranean sponges *Aplysina aerophoba* and *Aplysina cavernicola*. *FEMS Microbiol Ecol* **35**: 305–312.

Hentschel U, Usher KM & Taylor MW (2006) Marine sponges as microbial fermenters. *FEMS Microbiol Ecol* **55**: 167–177.

Hentschel U, Piel J, Degnam SM & Taylor MW (2012) Genomic insights into the marine sponge microbiome. *Nat Rev Microbiol* **10**: 641–654.

Hochmuth T & Piel J (2009) Polyketide synthases of bacterial symbionts in sponges - Evolution-based applications in natural products research. *Phytochemistry* **70**: 1841–1849.

Hoffmann M, Fischer M, Ottesen A, McCarthy PJ, Lopez JV, Brown EW & Monday SR (2010) Population dynamics of *Vibrio* spp. associated with marine sponge microcosms. *ISME J* **4**: 1608–1612.

Huelsenbeck JP & Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**: 754–755.

Ishii S & Sadowsky MJ (2009) Applications of the rep-PCR DNA fingerprinting technique to study microbial diversity, ecology and evolution. *Environ Microbiol* **11**: 733–740.

Joint I, Mühling M & Querellou J (2010) Culturing marine bacteria – an essential prerequisite for biodiscovery. *Microb Biotechnol* **3**: 564–575.

Kennedy J, Baker P, Piper C *et al.* (2009) Isolation and analysis of bacteria with antimicrobial activities from the marine sponge *Haliclona simulans* collected from Irish waters. *Mar Biotechnol* **11**: 384–396.

Kurahashi M & Yokota A (2007) *Endozoicomonas elysicola* gen. nov., sp. nov., a γ-proteobacterium isolated from the sea slug *Elysia ornata*. *Syst Appl Microbiol* **30**: 202–206.

Lafi FF, Garson MJ & Fuerst JA (2005) Culturable bacterial symbionts isolated from two distinct sponge species (*Pseudoceratina clavata* and *Rhabdastrella globostellata*) from the Great Barrier Reef display similar phylogenetic diversity. *Microb Ecol* **50**: 213–220.

Lee OO, Wang Y, Yang J, Lafi FF, Al-Suwailem A & Qian P-Y (2011) Pyrosequencing reveals highly diverse and species-specific microbial communities in sponges from the Red Sea. *ISME J* **5**: 650–664.

Liu YH, Jung JH & Zhang S (2006) Indole alkaloids from a sponge *Sarcotragus* species. *Biochem Syst Ecol* **34**: 453–456.

Liu Y, Ji H, Zhang S, Jung JH & Xu T (2008) Trisoxazole macrolides from the sponge *Sarcotragus* species. *Chem Nat Compd* **44**: 140–141.

Ludwig W, Strunk O, Westram R *et al.* (2004) ARB: a software environment for sequence data. *Nucleic Acids Res* **32**: 1363–1371.

Maldonado M, Ribes M & van Duyl FC (2012) Nutrient fluxes through sponges: biology, budgets, and ecological

implications. *Advances in Marine Biology*, **Vol. 62** (Becerro MA, Uriz MJ, Maldonado M & Turon X, eds), pp. 113–182. Academic Press, London.

Mansson M, Gram L & Larsen TO (2011) Production of bioactive secondary metabolites by marine *Vibrionaceae*. *Mar Drugs* **9**: 1440–1468.

Martin B, Humbert O, Camara M *et al.* (1992) A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae*. *Nucleic Acids Res* **20**: 3479–3483.

Menezes CBA, Bonugli-Santos RC, Miqueletto PB *et al.* (2010) Microbial diversity associated with algae, ascidians and sponges from the north coast of São Paulo state, Brazil. *Microbiol Res* **165**: 466–482.

Mitova M, Tommonaro G & De Rosa S (2003) A novel cyclopeptide from a bacterium associated with the marine sponge *Ircinia muscarum*. *Z Naturforsch* **58c**: 740–745.

Mitova M, Popov S & De Rosa S (2004) Cyclic peptides from a *Ruegeria* strain of bacteria associated with the sponge *Suberites domuncula*. *J Nat Prod* **67**: 1178–1181.

Muscholl-Silberhorn A, Thiel V & Imhoff JF (2008) Abundance and bioactivity of cultured sponge-associated bacteria from the mediterranean sea. *Microb Ecol* **55**: 94–106.

Nithyanand P, Indhumathi T, Ravi A & Pandian S (2011) Culture independent characterization of bacteria associated with the mucus of the coral *Acropora digitifera* from the Gulf of Mannar. *World J Microbiol Biotechnol* **27**: 1399–1406.

Nylander JAA (2008) MrModeltest 2.3. Available at http://www.abc.se/~nylander/mrmodeltest2/mrmodeltest2.html

O'Halloran JA, Barbosa TM, Morrissey JP, Kennedy J, O'Gara F & Dobson ADW (2011) Diversity and antimicrobial activity of *Pseudovibrio* spp. from Irish marine sponges. *J Appl Microbiol* **110**: 1495–1508.

Oku N, Adachi K, Matsuda S, Kasai H, Takatsuki A & Shizuri Y (2008) Ariakemicins A and B, novel polyketide-peptide antibiotics from a marine gliding bacterium of the genus *Rapidithrix*. *Org Lett* **10**: 2481–2484.

Piel J (2004) Metabolites from symbiotic bacteria. *Nat Prod Rep* **21**: 519–538.

Piel J, Hui D, Wen G, Butzke D, Platzer M, Fusetani N & Matsunaga S (2004) Antitumor polyketide biosynthesis by an uncultivated bacterial symbiont of the marine sponge *Theonella swinhoei*. *PNAS* **101**: 16222–16227.

Pires FR (2007) Padrões de distribuição e taxonomia para os Porifera da região central do Algarve. Master Thesis. Algarve University, Faro.

Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J & Glöckner FO (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* **35**: 7188–7196.

Redmond MC, Valentine DL & Sessions AL (2010) Identification of novel methane-, ethane-, and propane-oxidizing bacteria at marine hydrocarbon seeps by stable isotope probing. *Appl Environ Microbiol* **76**: 6412–6422.

Rifai S, Fassouane A, El-Abbouyi A, Wardani A, Kijjoa A & Van Soest R (2005a) Screening of antimicrobial activity of marine sponge extracts. *J Mycol Med* **15**: 33–38.

Rifai S, Fassouane A, Pinho PM, Kijjoa A, Nazareth N, Nascimento MS & Herz W (2005b) Cytotoxicity and inhibition of lymphocyte proliferation of fasciculatin, a linear furanosesterterpene isolated from *Ircinia variabilis* collected from the Atlantic Coast of Morocco. *Mar Drugs* **3**: 15–21.

Rodríguez F, Oliver JL, Marín A & Medina JR (1990) The general stochastic model of nucleotide substitution. *J Theor Biol* **142**: 485–501.

Rodríguez-Valera F (2002) Approaches to prokaryotic biodiversity: a population genetics perspective. *Environ Microbiol* **4**: 628–633.

Ronquist F & Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.

Rosenberg E, Koren O, Reshef L, Efrony R & Zilber-Rosenberg I (2007) The role of microorganisms in coral health, disease and evolution. *Nat Rev Microbiol* **5**: 355–362.

Santos OCS, Pontes P, Santos JFM, Muricy G, Giambiagi-deMarval M & Laport MS (2010) Isolation, characterization and phylogeny of sponge-associated bacteria with antimicrobial activities from Brazil. *Res Microbiol* **161**: 604–612.

Schirmer A, Gadkari R, Reeves CD, Ibrahim F, DeLong EF & Hutchinson CR (2005) Metagenomic analysis reveals diverse polyketide synthase gene clusters in microorganisms associated with the marine sponge *Discodermia dissoluta*. *Appl Environ Microbiol* **71**: 4840–4849.

Schloss PD & Handelsman J (2005) Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Appl Environ Microbiol* **71**: 1501–1506.

Schloss PD, Westcott SL, Ryabin T *et al.* (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* **75**: 7537–7541.

Schmitt S, Weisz JB, Lindquist N & Hentschel U (2007) Vertical transmission of a phylogenetically complex microbial consortium in the viviparous sponge *Ircinia felix*. *Appl Environ Microbiol* **73**: 2067–2078.

Schmitt S, Tsai P, Bell J *et al.* (2012) Assessing the complex sponge microbiota: core, variable and species-specific bacterial communities in marine sponges. *ISME J* **6**: 564–576.

Schneemann I, Nagel K, Kajahn I, Labes A, Wiese J & Imhoff JF (2010) Comprehensive investigation of marine actinobacteria associated with the sponge *Halichondria panicea*. *Appl Environ Microbiol* **76**: 3702–3714.

Shen YC, Lo KL, Lin YC, Khalil AT, Kuo YH & Shih PS (2006) Novel linear C-22-sesterterpenoids from sponge *Ircinia formosana*. *Tetrahedron Lett* **47**: 4007–4010.

Shen YC, Shih PS, Lin YS, Lin YC, Kuo YH, Koo YC & Khalil AT (2009) Irciformonins E-K, C-22-Trinorsesterterpenoids from the sponge *Ircinia formosana*. *Helv Chim Acta* **92**: 2101–2110.

Shieh WY, Lin YT & Jean WD (2004) *Pseudovibrio denitrificans* gen. nov., sp nov., a marine, facultatively anaerobic, fermentative bacterium capable of denitrification. *Int J Syst Evol Microbiol* **54**: 2307–2312.

Siegl A & Hentschel U (2010) PKS and NRPS gene clusters from microbial symbiont cells of marine sponges by whole genome amplification. *Environ Microbiol Rep* **2**: 507–513.

Siegl A, Kamke J, Hochmuth T, Piel J, Richter M, Liang CG, Dandekar T & Hentschel U (2011) Single-cell genomics reveals the lifestyle of *Poribacteria*, a candidate phylum symbiotically associated with marine sponges. *ISME J* **5**: 61–70.

Simister RL, Deines P, Botté ES, Webster NS & Taylor MW (2012) Sponge-specific clusters revisited: a comprehensive phylogeny of sponge-associated microorganisms. *Environ Microbiol* **14**: 517–524.

Sipkema D, Schippers K, Maalcke WJ, Yang Y, Salim S & Blanch HW (2011) Multiple approaches to enhance the cultivability of bacteria associated with the marine sponge *Haliclona* (gellius) sp. *Appl Environ Microbiol* **77**: 2130–2140.

Stabili L, Cardone F, Alifano P, Tredici SM, Piraino S, Corriero G & Gaino E (2012) Epidemic mortality of the sponge *Ircinia variabilis* (Schmidt, 1862) associated to proliferation of a vibrio bacterium. *Microb Ecol* **64**: 802–813.

Stamatakis A (2006) RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**: 2688–2690.

Staunton J & Weissman KJ (2001) Polyketide biosynthesis: a millennium review. *Nat Prod Rep* **18**: 380–416.

Sudek S, Lopanik NB, Waggoner LE, Hildebrand M, Anderson C, Liu H, Patel A, David H, Sherman DH & Haygood MG (2007) Identification of the putative bryostatin polyketide synthase gene cluster from "Candidatus Endobugula sertula", the uncultivated microbial symbiont of the marine bryozoan *Bugula neritina*. *J Nat Prod* **70**: 67–74.

Taylor MW, Radax R, Steger D & Wagner M (2007) Sponge-associated microorganisms: evolution, ecology and biotechnological potential. *Microbiol Mol Biol Rev* **71**: 295–347.

Teta R, Gurgui M, Helfrich EJN, Künne S, Schneider A, Van Echten-Deckert G, Mangoni A & Piel J (2010) Genome mining reveals trans-AT polyketide synthase directed antibiotic biosynthesis in the bacterial phylum *Bacteroidetes*. *ChemBioChem* **11**: 2506–2512.

Thiel V & Imhoff JF (2003) Phylogenetic identification of bacteria with antimicrobial activities isolated from Mediterranean sponges. *Biomol Eng* **20**: 421–423.

Thomas T, Rusch D, DeMaere MZ, Yung PY, Lewis M, Halpern A, Heidelberg KB, Egan S, Steinberg PD & Kjelleberg S (2010) Functional genomic signatures of sponge bacteria reveal unique and shared features of symbiosis. *ISME J* **4**: 1557–1567.

Thompson FL, Iida T & Swings J (2004) Biodiversity of Vibrios. *Microbiol Mol Biol Rev* **68**: 403–431.

Touchon M, Hoede C, Tenaillon O *et al.* (2009) Organised genome dynamics in the *Escherichia coli* species results in highly diverse adaptive paths. *PLoS Genet* **5**: e1000344.

Vacelet J (1970) Description de cellules a bactéries intranucléaires chez des éponges *Verongia*. *J Microsc* **9**: 333–346.

Vacelet J (1975) Étude en microscopie électronique de l'association entre bactéries et spongiaires du genre *Verongia* (Dictyoceratida). *J Microsc Biol Cell* **23**: 271–288.

Vacelet J & Donadey C (1977) Electron microscope study of the association between some sponges and bacteria. *J Exp Mar Biol Ecol* **30**: 301–314.

Van Elsas JD, Semenov AV, Costa R & Trevors JT (2011) Survival of *Escherichia coli* in the environment - fundamental and public health aspects. *ISME J* **5**: 173–183.

Versalovic J, Schneider M, de Bruijn FJ & Lupski JR (1994) Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods Mol Cell Biol* **5**: 25–40.

Wang N, Song J, Jang KH, Lee HS, Li X, Oh KB & Shin J (2008) Sesterterpenoids from the sponge *Sarcotragus* sp. *J Nat Prod* **71**: 551–557.

Webster NS & Hill RT (2001) The culturable microbial community of the Great Barrier Reef sponge *Rhopaloeides odorabile* is dominated by an alpha-Proteobacterium. *Mar Biol* **138**: 843–851.

Webster NS & Taylor MW (2012) Marine sponges and their microbial symbionts: love and other relationships. *Environ Microbiol* **14**: 335–346.

Webster NS, Taylor MW, Behnam F, Lucker S, Rattei T, Whalan S, Horn M & Wagner M (2010) Deep sequencing reveals exceptional diversity and modes of transmission for bacterial sponge symbionts. *Environ Microbiol* **12**: 2070–2082.

Weisburg WG, Barns SM, Pelletier DA & Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**: 697–703.

Weisz J, Lindquist N & Martens C (2008) Do associated microbial abundances impact marine demosponge pumping rates and tissue densities? *Oecologia* **155**: 367–376.

Wichels A, Würtz S, Döpke H, Schütt C & Gerdts G (2006) Bacterial diversity in the breadcrumb sponge *Halichondria panicea* (Pallas). *FEMS Microbiol Ecol* **56**: 102–118.

Wilkinson CR (1978) Microbial associations in sponges II. Numerical analysis of sponge and water bacterial populations. *Mar Biol* **49**: 169–176.

Zan J, Fuqua C & Hill RT (2011) Diversity and functional analysis of luxS genes in Vibrios from marine sponges *Mycale laxissima* and *Ircinia strobilina*. *ISME J* **5**: 1505–1516.

Zhang W, Li Z, Miao X & Zhang F (2009a) The screening of antimicrobial bacteria with diverse novel nonribosomal peptide synthetase (NRPS) genes from South China Sea sponges. *Mar Biotechnol* **11**: 346–355.

Zhang W, Zhang F, Li Z, Miao X, Meng Q & Zhang X (2009b) Investigation of bacteria with polyketide synthase genes and antimicrobial activity isolated from South China Sea sponges. *J Appl Microbiol* **107**: 567–575.