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# Similar sponge-associated bacteria can be acquired via both vertical and horizontal transmission

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## Summary

**Marine sponges host diverse communities of microorganisms that are often vertically transmitted from mother to oocyte or embryo. Horizontal transmission has often been proposed to co-occur in marine sponges, but the mechanism is poorly understood. To assess the impact of the mode of transmission on the microbial assemblages of sponges, we analysed the microbiota in sympatric sponges that have previously been reported to acquire bacteria via either vertical (*Corticium candelabrum* and *Crambe crambe*) or horizontal transmission (*Petrosia ficiformis*). The comparative study was performed by polymerase chain reaction-denaturing gradient gel electrophoresis and pyrosequencing of barcoded PCR-amplified 16S rRNA gene fragments. We found that *P. ficiformis* and *C. candelabrum* each harbour their own species-specific bacteria, but they are similar to other high-microbial-abundance sponges, while the low-microbial-abundance sponge *C. crambe* hosts microbiota of a very different phylogenetic signature. In addition, nearly 50% of the reads obtained from *P. ficiformis* were most closely related to bacteria that were previously reported to be vertically transmitted in other sponges and comprised vertical–horizontal transmission phylogenetic clusters (VHT clusters). Therefore, our results provide evidence for the hypothesis that similar sponge-associated bacteria can be acquired via both vertical and horizontal transmission.**

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## Introduction

'When it comes to sex, sponges probably win the prize for variety' is the claim by Brusca and Brusca (1990) in a book chapter about reproduction of marine sponges. One aspect of sexual reproduction of sponges that was not dealt with in their treatise, and even makes reproduction of sponges more varied, is the incorporation of microorganisms inside egg cells or other reproductive stages. Such vertical transmission has been observed for all classes of the Porifera (e.g. Ereskovsky *et al.*, 2005). The presence of bacteria in sponge reproductive stages has been repeatedly reported (Uriz *et al.*, 2001; 2012; Ereskovsky *et al.*, 2005; Usher *et al.*, 2005; Enticknap *et al.*, 2006; De Caralt *et al.*, 2007; Schmitt *et al.*, 2007; 2008; Sharp *et al.*, 2007; Lee *et al.*, 2009), but also archaea (Sharp *et al.*, 2007; Steger *et al.*, 2008) and yeast have been observed (Maldonado *et al.*, 2005). Thus, many sponge species ensure that their associated microorganisms, which are thought to be important partners for the sponge, will be available to their offspring. Vertical transmission (transmission via reproductive stages) of associated microorganisms is a feature that has also been observed in a number of other marine organisms, such as bivalves (Krueger *et al.*, 1996), ascidians, bryozoans, oligochaetes (Bright and Bulgheresi, 2010) and insects (Hosokawa *et al.*, 2006), and it is often seen as a clear indicator for a symbiotic relationship between host and microbe. In addition, sponges predominantly harbour microorganisms that are mostly restricted to sponges (termed sponge-specific microorganisms) or in sponges and corals (termed sponge&coral-specific microorganisms), which implies that the host represents a selective niche for microorganisms (Simister *et al.*, 2012). Nevertheless, although there is a consensus that at least some associations between sponges and bacteria are mutually beneficial, the exact nature of many of these associations is still unclear.

Horizontal transmission (recruitment of microorganisms from the environment) has been proposed for sponges to

occur in addition to, or as an alternative to vertical transmission (Taylor *et al*., 2007). However, little experimental evidence is available to support this hypothesis. Issues that obscure a clear picture of the transmission mechanism are:

(i) Presence of bacteria in sponge larvae is usually mentioned in ultrastructure and molecular studies, while in contrast, the absence of bacteria in reproductive stages is rarely reported.
(ii) Sponges may combine vertical and horizontal transmission, in such way that some associated microbes are vertically transmitted, while others are horizontally transmitted (Taylor *et al*., 2007). It should be noted, however, that the few studies that have compared the microbial composition in both reproductive stages and adults indicated that where vertical transmission occurs, the majority of microbial species that are found in adult sponges are also present in their reproductive stages (De Caralt *et al*., 2007; Sharp *et al*., 2007; Schmitt *et al*., 2008; Lee *et al*., 2009; Bergman *et al*., 2011).
(iii) The same bacterial species that are vertically transmitted may also be acquired horizontally from the water column (Taylor *et al*., 2007).
(iv) Some bacteria that are detected in sponges may be present solely as part of the sponge diet or be the result of a temporal enrichment and do not make part of the stably associated microbiota.

Marine sponges seem particularly fit to acquire their associated microorganisms horizontally, because their high water pumping rates and efficient filtration system guarantee the encounter and capture of an estimated $10^{10}$ cells per day per ml sponge (Hill, 2004). It has been hypothesized that immune system-like proteins that have been found in sponges could play a role in the selection of bacteria (Müller and Müller, 2003). In addition, sponge-associated bacteria are particularly rich in ankyrin and tetratricopeptide repeats that could play a role in the specific interactions with sponge cells (Thomas *et al*., 2010), however, the mechanism is yet poorly understood.

*Petrosia ficiformis* is the only sponge species known for which oocytes, spermatocytes, embryos and larvae have been extensively studied by electron microscopy and for which it has been repeatedly reported that no bacteria were observed in the sponge's reproductive stages (Lepore *et al*., 1995; Maldonado and Riesgo, 2009). This led to the conclusion that microbes are acquired from the ambient water by each new generation of *P. ficiformis* juveniles (Maldonado and Riesgo, 2009).

Our aim was to investigate to what extent the associated microbial community is shaped by the mode of transmission. To this end, we characterized the associated microbiota of the high-microbial-abundance sponge[1] *P. ficiformis* in detail and compared it to the associated microbiota of two sympatric sponge species: the high-microbial-abundance sponge *C. candelabrum* and the low-microbial-abundance[1] sponge *C. crambe*. For both of the latter species, the majority of the associated bacteria are vertically transmitted (Galera *et al*., 2000; Uriz *et al*., 2001; De Caralt *et al*., 2007).

## Results and discussion

### *Microbial community comparison*

The microbial communities inhabiting the marine sponges *P. ficiformis*, *C. candelabrum* and *C. crambe* were compared with the microbial community in the surrounding seawater. An initial comparison of bacterial community composition in all sponge and water using denaturing gradient gel electrophoresis (DGGE) profiling of polymerase chain reaction (PCR)-amplified 16S rRNA gene fragments, revealed that the three sponge species all have their own species-specific bacterial fingerprint that is also different from the surrounding seawater (Fig. S1). In order to test for significant differences between DGGE profiles for different sample types, analyses of similarity (ANOSIM, Clarke, 1993) were performed on binary matrixes using the one-way ANOSIM function. R values for all sample type comparisons were equal to one, indicating that indeed all sample types were different. Associated *P*-values amounted to 0.008, which is the maximum significance that can be obtained considering the actual number of permutations. Only for the comparison between *C. candelabrum* and seawater the *P*-value was 0.029 due to the lower number of permutations (35) as DNA from the fifth *C. candelabrum* specimen could not be amplified. Within sample type similarity was calculated by SIMPER (Table S1). Bacterial profiles of the four *C. candelabrum* specimens were most similar with an average similarity of 94.3%. In *P. ficiformis* intraspecific variation was higher than in *C. candelabrum* (average similarity 71.7%), while *C. crambe* specimens shared only three consistent DGGE bands (average similarity 69.3%) of which two were also present in the seawater. This was confirmed by sequencing of excised DGGE bands (sw63 = Cr62 and sw10 = Cr49).

Two representative individuals of *P. ficiformis*, *C. candelabrum*, *C. crambe* and seawater were selected for a detailed study of microbial diversity by pyrosequencing of partial 16S rRNA genes. Qualified non-chimeric read

[1]High-microbial-abundance sponges are sponges that typically contain $10^8$–$10^{10}$ bacteria per gram of sponge wet weight, while low-microbial-abundance sponges typically contain bacterial densities of $10^5$–$10^6$ bacteria per gram sponge wet weight, which is in the range of seawater (Hentschel *et al*., 2006).

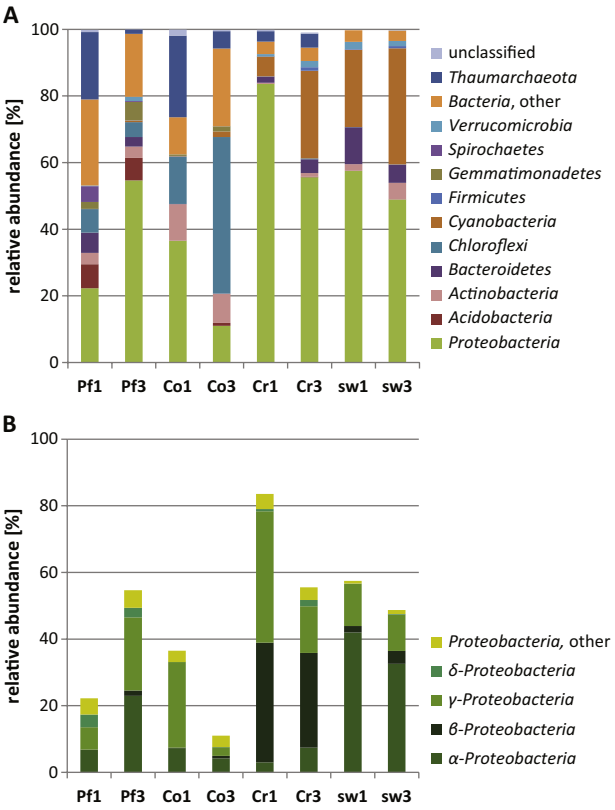



**Fig. 1.** A. Taxonomic distribution of bacterial and archaeal reads retrieved from different sponge species and seawater at phylum level using the RDP classifier with a confidence threshold of 80%. The taxonomic affiliation of unclassified OTUs that represented more than 1% of the reads in individual samples was resolved by importing these OTUs by parsimony in a Bayesian tree of related sequences (see Fig. 4). Only phyla that represent more than 1% of the reads in at least one of the samples are included. 'Pf' refers to *P. ficiformis* individuals, 'Co' to *C. candelabrum*, 'Cr' to *C. crambe* and 'sw' to seawater samples. Total non-chimeric read numbers (including phyla that represent less than 1% of the reads in all samples) were: Pf1 9046, Pf3 9225, Co1 11493, Co3 9709, Cr1 8303, Cr3 6452, sw1 10524 and sw3 9510. B. The classes of Proteobacteria as a percentage of the total number of reads. ε-Proteobacteria represented less than 0.2% of the reads in all samples and are not included. 'Proteobacteria, other' are Proteobacteria that were not classified to the class level by RDP at the confidence threshold of 80%.

numbers ranged from 6452 to 11 493 reads per sample with an average read length of 384 bp, and the rarefaction curves showed that all samples were sequenced at sufficient depth (Fig. S2) to discuss similarity and differences between samples for OTUs representing $>1\%$ of the reads. At a confidence threshold of 80%, 51 937 out of 74 262 qualified non-chimeric reads could be assigned to a known phylum using the Ribosomal Database Project classifier. Proteobacteria was the dominant phylum in all sponge species, with the exception of one individual of *C. candelabrum*, for which Chloroflexi represented the largest number of reads (Fig. 1). Alpha (α)- and gamma (γ)-Proteobacteria were the most prominent classes in *P. ficiformis* and *C. candelabrum*, while beta (β)- and γ-Proteobacteria were the predominant proteobacterial classes in *C. crambe*. At the phylum level the high-microbial-abundance sponges *P. ficiformis* and *C. candelabrum* showed similar microbial profiles except that Acidobacteria were abundant in *P. ficiformis*, whereas they were present in very low numbers in *C. candelabrum*. Both in the low-microbial-abundance sponge *C. crambe* and seawater, a large proportion of the reads was affiliated with Cyanobacteria, and together with Proteobacteria they accounted for $84\% \pm 3.9\%$ of all reads in these samples.

In correspondence to the phylum-level observation, at the approximate species level the microbial profiles of *C. crambe* and surrounding seawater shared a substantial percentage (4.8%) of operational taxonomic units (OTUs; Fig. 2). The similarity at the phylum level between *P. ficiformis* and *C. candelabrum* was not reflected at the approximate species level as both sponge species possessed their own specific microbiota (1.4% of shared OTUs). In addition, only a limited number of OTUs was shared between *P. ficiformis*, *C. candelabrum* and seawater-derived OTUs (0.65% and 0.63%, respectively). Comparison of sequences derived from DGGE-bands with OTUs obtained by pyrosequencing showed that

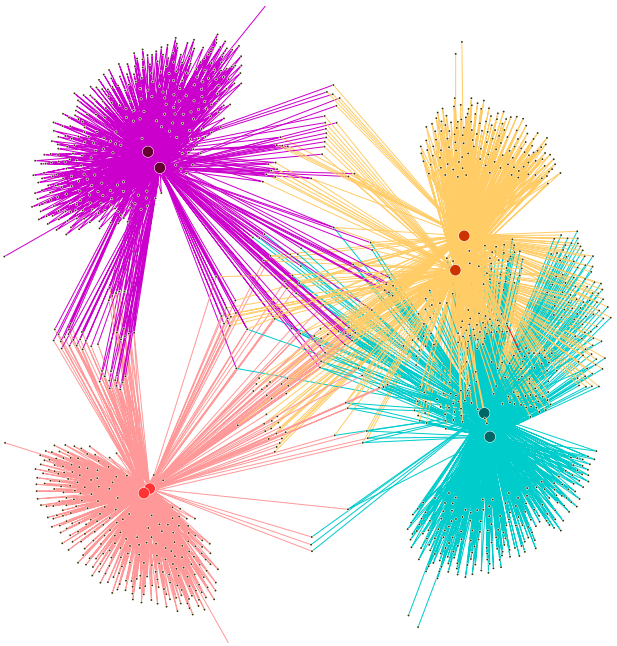


**Fig. 2.** Microbial profiles of OTUs in two *P. ficiformis* individuals (purple dots), two *C. candelabrum* individuals (pink dots), two *C. crambe* individuals (orange dots) and two seawater samples (blue dots). The distance between samples is their non-metric multidimensional scaling distance, which is correlated to differences in weighted OTU profiles. The small black dots represent individual OTUs that are either shared between samples or present in single samples.

nearly all sequences found through DGGE were also recovered with pyrosequencing (Table S2).

Operational taxonomic units representing at least 1% of the reads in one of the samples (69 OTUs) were selected for further phylogenetic analysis. These OTUs accounted for 60.2% ± 6.4% of the total number of reads in all sponge and water samples. Most OTUs from the high-microbial-abundance sponges *P. ficiformis* and *C. candelabrum* were closely related to sequences derived from other sponges (Fig. 3, Fig. S3), but only two OTUs represented more than 1% of the reads in both *P. ficiformis* and *C. candelabrum*. One of these OTUs (OTU8459) makes part of a sponge-specific clade within the family Rhodospirillaceae (α-Proteobacteria), a group of mainly phototrophic sulfur-oxidizing bacteria (Sander and Dahl, 2009), for which no reads were recovered from the seawater. It has been hypothesized that a bacterial sulfur cycle exists in sponges in which both sulfur-oxidizing and sulfur-reducing bacteria cooperate (Hoffmann *et al*., 2005). We cannot confirm this hypothesis as only a few assigned reads (10) in *P. ficiformis* and *C. candelabrum* were associated to known proteobacterial sulfate-reducing bacteria (Muyzer and Stams, 2008), such as Desulfobacterales and Desulfovibrionales, while no reads were obtained for known sulfate-reducing genera within Firmicutes, Nitrospirae, Euryarchaeota and Crenarchaeota. It cannot be excluded that detailed phylogenetic analysis of unclassified OTUs with relative abundance below 1% would reveal some of those clades; however, their low abundance might argue against high-functional relevance within the sponge holobiont. The other OTU that was found in both *P. ficiformis* and *C. candelabrum* (OTU10083) is a member of the Thaumarchaeota that is often encountered in marine sponges (Holmes and Blanch, 2007). This OTU was also detected in *C. crambe* (1%/3.5% of the reads in Cr1/Cr3, respectively) and in seawater, albeit in low numbers (0%/0.07% of the reads). It is most closely related to the giant thaumarchaeon '*Candidatus* Giganthauma insulaporcus' that lives associated with bacteria with sulfur-oxidizing potential (Muller *et al*., 2010). However, the low phylogenetic resolution of the archaeal 16S rRNA gene (Schleper *et al*., 1998), in combination with the short read length of sequences obtained in this study, requires a cautious interpretation of the data, especially since no giant archaea were reported from electron microscopy studies of *P. ficiformis* and *C. candelabrum* (Vacelet and Donadey, 1977; De Caralt *et al*., 2007; Maldonado, 2007).

### Petrosia ficiformis-*associated microbiota*

Proteobacteria represented the majority of reads that could be classified in both sequenced *P. ficiformis* individuals, but with considerable variability [22% and 55% in *P. ficiformis* 1 (Pf1) and 3 (Pf3) respectively]. The reduced number of Proteobacteria in Pf1 was accompanied by an opposite trend for Thaumarchaeota (20.2% in Pf1 and 1.3% in Pf3), while the number of reads in other phyla were quite similar for the two specimens (Fig. 1). It could be speculated that microbial partners are selected by the host based on their biological functions or presence in the surrounding seawater at the time of acquisition rather than on phylogenetic identity, which may lead to higher intraspecific variation of associated microbiota that are acquired from the environment compared with vertically transferred microbiota. It is known that phylogenetically closely related sponge species of the genus *Tethya* each harbour phylogenetically very different phototrophic symbionts (cyanobacteria, algae and stramenopiles) each of which likely have similar roles in the respective holobionts (Sipkema and Blanch, 2010). In addition, for two high-microbial-abundance sponges, it was shown that such functional (rather than phylogenetic) similarity also exists for uptake of dissolved organic carbon and assimilation of ammonia, nitrate and nitrite (Fan *et al*., 2012; Ribes *et al*., 2012). It is, however, unknown whether these selective associations of functionally similar microorganisms also occur within individuals belonging to the same species. While the proteobacteria comprised many OTUs in *P. ficiformis*, nearly all Thaumarchaeota-related reads were grouped in a single OTU (OTU3772) that is closely related to the globally distributed ammonia-oxidizing *Nitrosopumilus maritimus*, one of the few isolated Thaumarchaeota (Könneke *et al*., 2005).

Other prominent phyla in *P. ficiformis* were Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi and Gemmatimonadetes. Our data roughly correlate to previous studies of bacterial diversity in *P. ficiformis* that were based on lower numbers of reads per sample (Schmitt *et al*., 2012; Blanquer *et al*., 2013; Burgsdorf *et al*., 2014), and the phyla encountered are typically those which are frequently found in high-microbial-abundance sponges (Hentschel *et al*., 2006). This typical sponge-associated microbial profile was confirmed at the lower phylogenetic level as all but one OTUs that represented more than 1% of the reads from *P. ficiformis* were either part of clusters exclusively representing sequences from sponges and corals or had sponge-derived sequences among their nearest neighbours (Fig. 3). For example, the bacterial OTU that represented most of the reads in *P. ficiformis* (OTU6992) belongs to a clade of Acidobacteria that has been exclusively recovered from a wide range of sponges. Lower numbers of reads were recovered from the phyla Spirochaetes, Verrucomicrobia, Cyanobacteria, Firmicutes, candidate division OD1, Planctomycetes and Nitrospira (in order of prevalence). The low number of cyanobacterial sequences may seem surprising as it is known that *Synechoccus feldmanni*

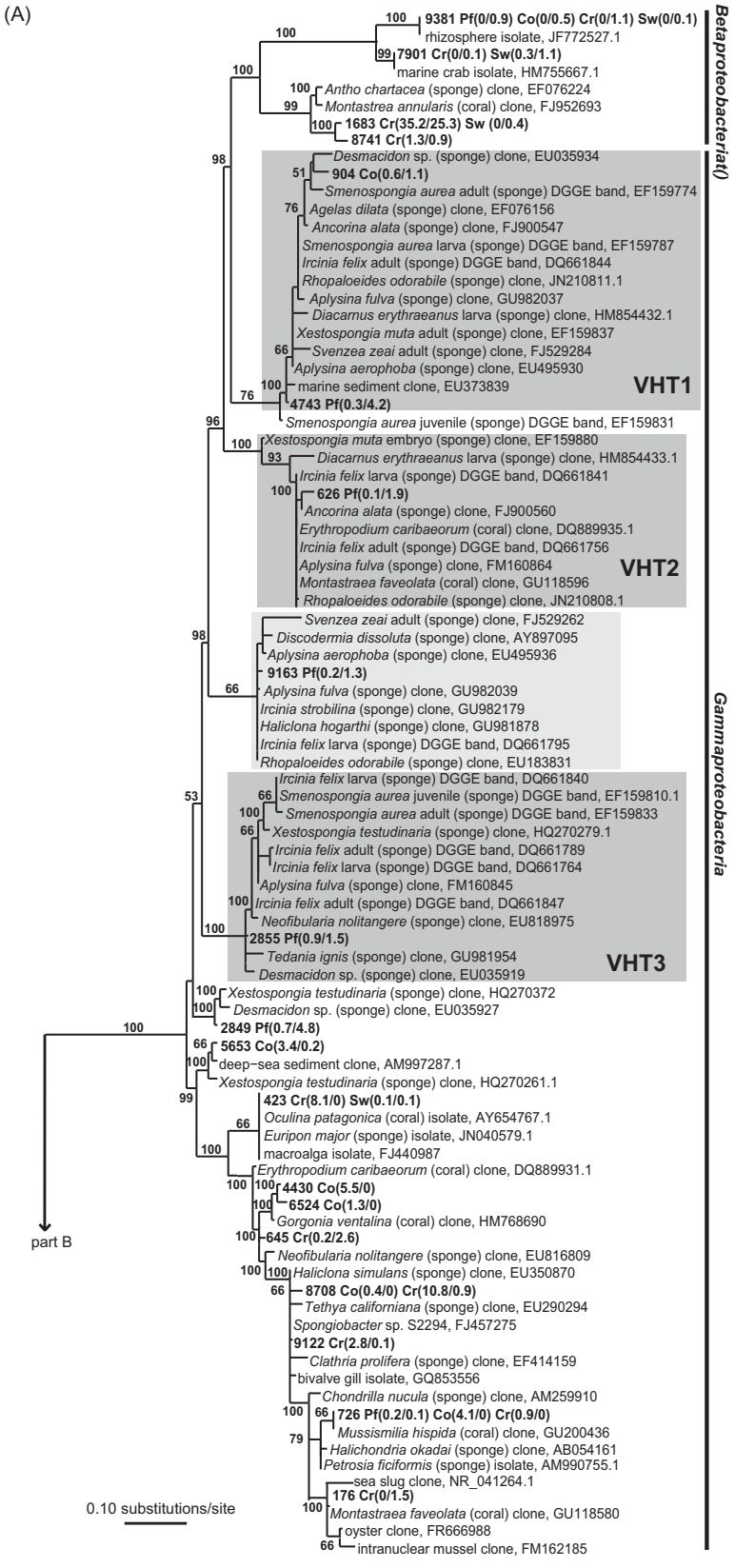



**Fig. 3.** A–D. Bayesian phylogram based on 16S rRNA gene sequences of OTUs representing more than 1% of the reads in *P. ficiformis* and *C. candelabrum* and their nearest neighbours. Operational taxonomic units that include reads from *C. crambe* and seawater are only included if they also represent more than 1% of the reads in *P. ficiformis* or *C. candelabrum*. Operational taxonomic units from this study are in bold, and 'Pf' indicates that the OTU contains reads derived from *P. ficiformis* individuals, 'Co' from *C. candelabrum*, 'Cr' from *C. crambe* and 'sw' from seawater. Numbers in parenthesis after the sample type indication refer to the percentage of reads that are included in the corresponding OTU per sample [9381 Pf(0/0.9) means that 0% of the reads of Pf1 and 0.9% of the reads of Pf3 are included in OTU9381]. Dark grey boxes indicate VHT clusters that are supported by posterior probability (PP) values > 95%. These, clusters comprise OTUs from both Pf adults and sequences derived from other sponge species larvae or embryos. Light-grey boxes indicate potential VHT clusters that have PP values < 95%. Numbers above or below the branches correspond to PP values of the Bayesian analysis. Nodes with PP values of < 50 are not indicated. The complete phylogenetic analysis including all *C. crambe* and seawater OTUs that represent more than 1% of the reads is presented in supplementary Fig. S3. (A) β- and γ-Proteobacteria, (B) α- and δ-Proteobacteria, (C) Acidobacteria, Actinobacteria, Gemmatimonadetes, δ-Proteobacteria and Bacteroidetes and (D) Chloroflexi, Cyanobacteria, Spirochaetes and Thaumarchaeota.

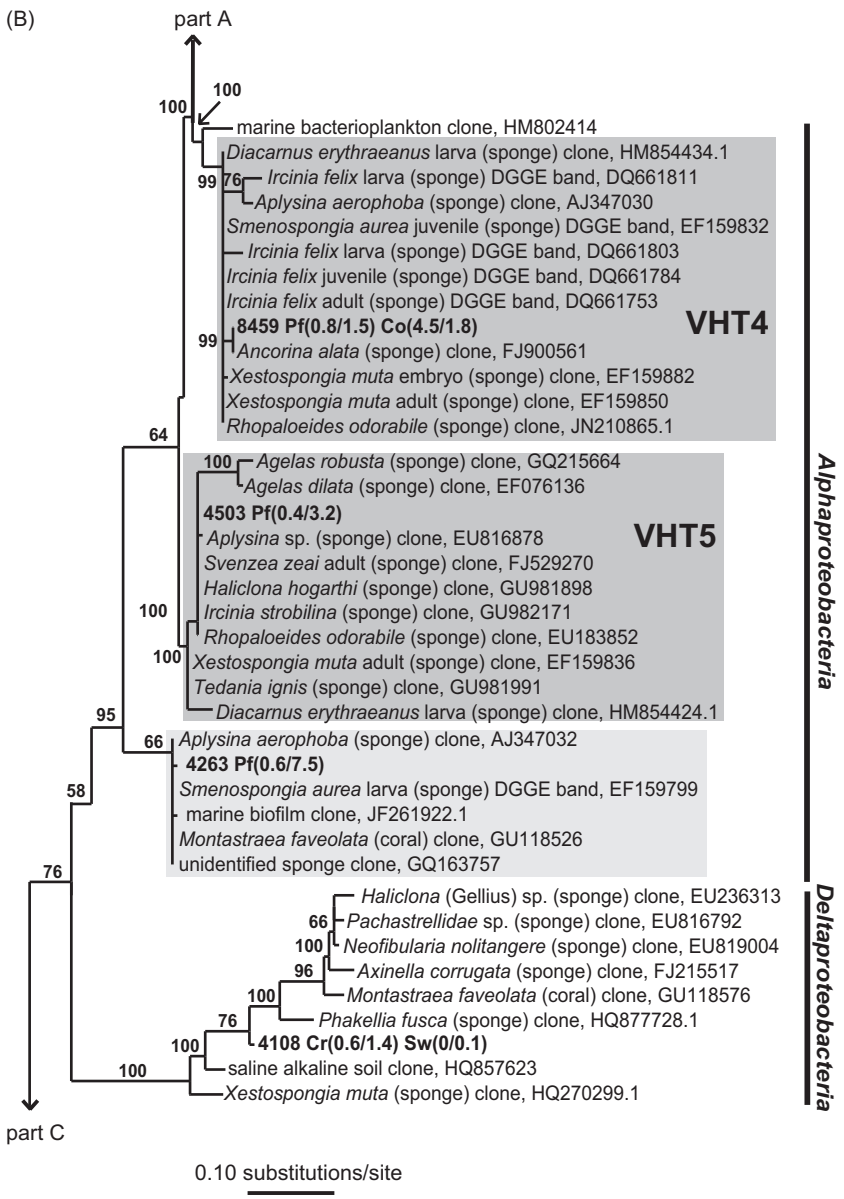



**Fig. 3.** *cont.*

is abundant in what is called the 'symbiocortex' of *P. ficiformis* individuals growing in the light (Steindler *et al*., 2007; Burgsdorf *et al*., 2014). It should be noted, however, that for our study we only analysed the sponge's interiors to avoid seawater contaminations as much as possible, and the purple-red cortex was removed before DNA was extracted.

### *Impact of the transmission strategy on the sponge-associated microbiota*

At the phylum level, the microbial fingerprints of *P. ficiformis* (horizontal transmission) and *C. candelabrum* (vertical transmission) were found to be rather similar. This similarity is not related to the sampling site as *C. crambe*, also collected from the same habitat, has a completely different microbial profile. At the approximate species level, however, only two OTUs (> 1% of the reads) were shared between *P. ficiformis* and *C. candelabrum*, even though it should be noted that in both cases their microbiota was dominated by microorganisms that were so far mainly found in sponges and corals.

At first sight, this observation might suggest that two classes of sponge-associated bacteria exist, one that is vertically and one that is horizontally transmitted. However, our data do not support this hypothesis as a large number of OTUs of *P. ficiformis* (horizontal) were closely related to sequences that were present in vertical transmission clusters (sequences found in adult and embryo/larvae) of the sponges *Smenospongia aurea*,

(C)

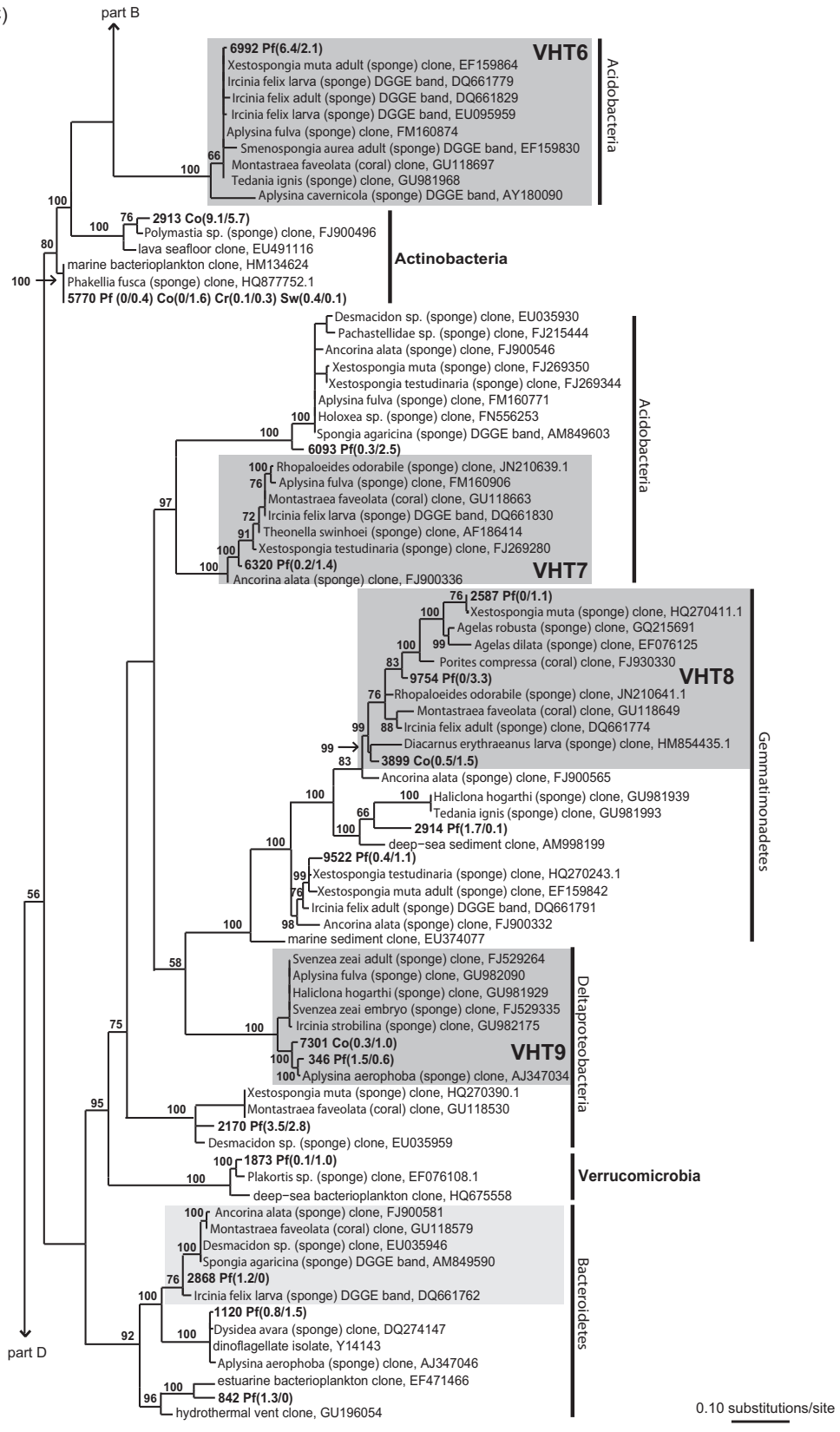
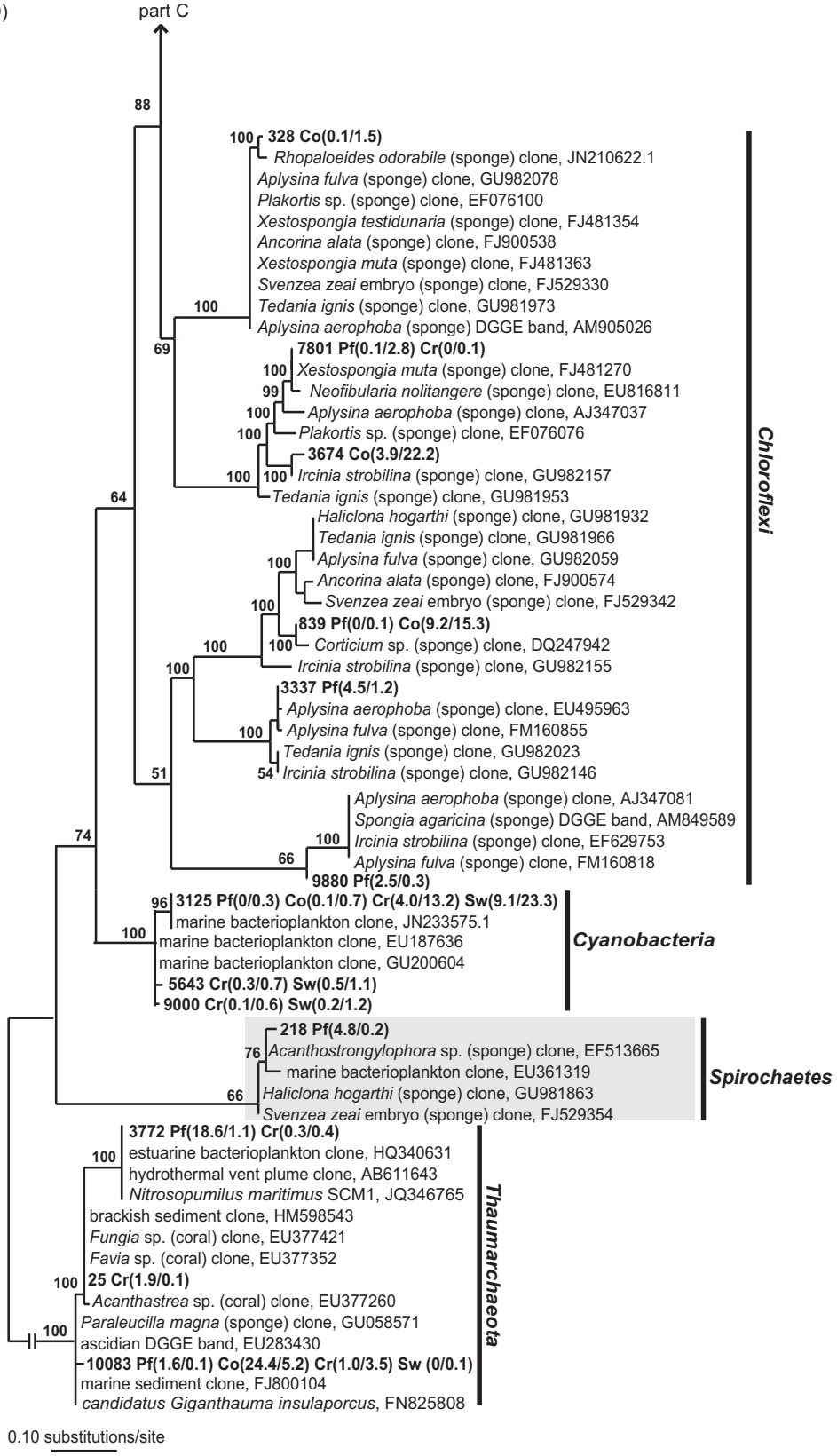

part B
6992 Pf(6.4/2.1)
VHT6
Xestospongia muta adult (sponge) clone, EF159864
Ircinia felix larva (sponge) DGGE band, DQ661779
Ircinia felix adult (sponge) DGGE band, DQ661829
Ircinia felix larva (sponge) DGGE band, EU095959
Aplysina fulva (sponge) clone, FM160874
Smenospongia aurea adult (sponge) DGGE band, EF159830
Montastraea faveolata (coral) clone, GU118697
Tedania ignis (sponge) clone, GU981968
Aplysina cavernicola (sponge) DGGE band, AY180090
Acidobacteria
2913 Co(9.1/5.7)
Polymastia sp. (sponge) clone, FJ900496
lava seafloor clone, EU491116
marine bacterioplankton clone, HM134624
Phakellia fusca (sponge) clone, HQ877752.1
5770 Pf (0/0.4) Co(0/1.6) Cr(0.1/0.3) Sw(0.4/0.1)
Actinobacteria
Desmacidon sp. (sponge) clone, EU035930
Pachastellidae sp. (sponge) clone, FJ215444
Ancorina alata (sponge) clone, FJ900546
Xestospongia muta (sponge) clone, FJ269350
Xestospongia testudinaria (sponge) clone, FJ269344
Aplysina fulva (sponge) clone, FM160771
Holoxea sp. (sponge) clone, FN556253
Spongia agaricina (sponge) DGGE band, AM849603
6093 Pf(0.3/2.5)
Acidobacteria
Rhopaloeides odorabile (sponge) clone, JN210639.1
Aplysina fulva (sponge) clone, FM160906
Montastraea faveolata (coral) clone, GU118663
Ircinia felix larva (sponge) DGGE band, DQ661830
Theonella swinhoei (sponge) clone, AF186414
Xestospongia testudinaria (sponge) clone, FJ269280
6320 Pf(0.2/1.4)
Ancorina alata (sponge) clone, FJ900336
VHT7
2587 Pf(0/1.1)
Xestospongia muta (sponge) clone, HQ270411.1
Agelas robusta (sponge) clone, GQ215691
Agelas dilata (sponge) clone, EF076125
Porites compressa (coral) clone, FJ930330
9754 Pf(0/3.3)
VHT8
Rhopaloeides odorabile (sponge) clone, JN210641.1
Montastraea faveolata (coral) clone, GU118649
Ircinia felix adult (sponge) clone, DQ661774
Diacarnus erythraeanus larva (sponge) clone, HM854435.1
3899 Co(0.5/1.5)
Ancorina alata (sponge) clone, FJ900565
Haliclona hogarthi (sponge) clone, GU981939
Tedania ignis (sponge) clone, GU981993
2914 Pf(1.7/0.1)
deep-sea sediment clone, AM998199
9522 Pf(0.4/1.1)
Xestospongia testudinaria (sponge) clone, HQ270243.1
Xestospongia muta adult (sponge) clone, EF159842
Ircinia felix adult (sponge) DGGE band, DQ661791
Ancorina alata (sponge) clone, FJ900332
marine sediment clone, EU374077
Gemmatimonadetes
Svenzea zeai adult (sponge) clone, FJ529264
Aplysina fulva (sponge) clone, GU982090
Haliclona hogarthi (sponge) clone, GU981929
Svenzea zeai embryo (sponge) clone, FJ529335
Ircinia strobilina (sponge) clone, GU982175
7301 Co(0.3/1.0)
346 Pf(1.5/0.6)
Aplysina aerophoba (sponge) clone, AJ347034
VHT9
Deltaproteobacteria
Xestospongia muta (sponge) clone, HQ270390.1
Montastraea faveolata (coral) clone, GU118530
2170 Pf(3.5/2.8)
Desmacidon sp. (sponge) clone, EU035959
1873 Pf(0.1/1.0)
Plakortis sp. (sponge) clone, EF076108.1
deep-sea bacterioplankton clone, HQ675558
Verrucomicrobia
Ancorina alata (sponge) clone, FJ900581
Montastraea faveolata (coral) clone, GU118579
Desmacidon sp. (sponge) clone, EU035946
Spongia agaricina (sponge) DGGE band, AM849590
2868 Pf(1.2/0)
Ircinia felix larva (sponge) DGGE band, DQ661762
1120 Pf(0.8/1.5)
Dysidea avara (sponge) clone, DQ274147
dinoflagellate isolate, Y14143
Aplysina aerophoba (sponge) clone, AJ347046
estuarine bacterioplankton clone, EF471466
842 Pf(1.3/0)
hydrothermal vent clone, GU196054
Bacteroidetes
part D
0.10 substitutions/site

**Fig. 3.** *cont.*

(D) part C

**Fig. 3.** *cont.*



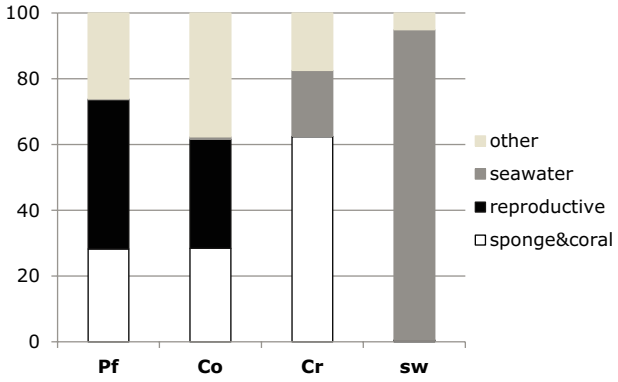



**Fig. 4.** All reads from OTUs that represent more than 1% of the reads in any of the samples were categorized into four groups according to the origin of their nearest neighbours. (i) Reads from OTUs with neighbours that were solely derived from other sponges and corals, (ii) neighbours that were derived from sponges and corals including sponge reproductive stages, (iii) neighbours that were solely derived from seawater and (iv) neighbours that were a mixture of the above-mentioned categories or from other sources. Samples were pooled per sample type (i.e. Pf = Pf1 + Pf3, etc.) and depicted as a percentage of the total number of reads in OTUs with more than 1% in the corresponding sample type.

*Xestospongia muta*, *Agelas wiedenmayeri* (Schmitt *et al*., 2008), *Ircinia felix* (Schmitt *et al*., 2007), *Svenzea zeai* (Lee *et al*., 2009) and *Diacarnus erythraeanus* (Bergman *et al*., 2011). Hence, we introduce vertical–horizontal transmission clusters (VHT clusters) that comprise sequences obtained from sponge reproductive stages and sequences obtained from *P. ficiformis* adults (Fig. 3). Partial 16S rRNA gene sequences of all members of each VHT cluster including those obtained in this study within each VHT cluster shared more than 95% identity (Table S3), and nodes of the clusters had Bayesian posterior probabilities larger than 95% (Fig. 3). At the identity level of 97%, most VHT clusters were maintained, which implies a high similarity between the bacteria present in these clusters (supplementary Table S3). Only VHT clusters 2 (γ-Proteobacteria) and 8 (Gemmatimonadetes) were not recovered at the 97% identity level.

When reads obtained from the three sponge species and seawater studied here, are categorised according to the origin of their nearest neighbours, it can be seen that approximately 45% of the reads of *P. ficiformis* is related to 16S rRNA gene sequences that have been obtained from reproductive stages from other sponges (Fig. 4). Approximately, 33% of the reads from *C. candelabrum* belong to this class, while nearly no reads obtained from *C. crambe* or seawater were related to sequences that were also obtained from sponge reproductive stages (0.1 and 0.01% of the reads respectively). It should be noted that most probably these percentages would actually be higher for all sample types when more studies would be performed on molecular characterisation of sponge reproductive stage-associated bacteria. Nevertheless, these data imply a selective enrichment of bacteria that belong to this class in *P. ficiformis*. Coral-derived sequences were also present in clusters containing sequences from sponge reproductive stage-associated bacteria, which suggests that the distinction of sponge-specific clusters on one hand and sponge&coral-specific clusters on the other hand (Simister *et al*., 2012) appears not to be related to the mode of transmission. The coral-derived sequences present in VHT clusters were obtained from the spawning coral species *Montastraea faveolata*, *Erythropodium caribaeorum* and *Porites compressa* (Fig. 3) that obtain their associated bacteria by horizontal transmission (Richmond and Hunter, 1990; Sharp *et al*., 2010). This indicates at least a part of the horizontally transmitted bacteria in corals are related to bacteria that have been found to be vertically (and also horizontally in our study) transmitted in sponges. Vertical transmission of bacterial communities in corals was only recently described for the brooding coral *Porites astreoides* (Sharp *et al*., 2012) and coral VHT clusters may be identified when more sequences of vertically transmitted bacteria in corals will be available.

Thus, these data suggest that similar associated bacteria can be obtained via vertical or horizontal transmission in sponges (and possibly corals). More sponge species that acquire their associated microbiota solely or mostly from the seawater should be studied to support this conclusion, but it is currently unknown which species these are. Bacteria that are both vertically and horizontally transmitted exist in insects. They are considered to be facultative symbionts and explain the incongruent evolutionary history of host and bacterial symbionts (Degnan *et al*., 2011; Gonella *et al*., 2012). A similar incongruence of host and bacteria phylogeny has been observed for sponges and their associated bacteria (Erpenbeck *et al*., 2002; Schmitt *et al*., 2008), which led to the hypothesis of a model of mixed vertical and horizontal transmission of bacteria in sponges (Schmitt *et al*., 2012) and highlight the importance of both sponge host-specific and environment specific factors (Erwin *et al*., 2012). Our results demonstrate for the first time the existence of clusters of sponge-specific and sponge&coral-specific bacteria that contain sequences from both vertically and horizontally transmitted bacteria and underpin the model proposed by Schmitt and colleagues (2012).

Horizontal transmission of sponge-associated bacteria requires that the bacteria are present in the environment where the sponges live. However, of all 27 OTUs that represented more than 1% of the reads in one of the *P. ficiformis* specimens, only three were also detected in surrounding seawater in miniscule numbers (Fig. 5). This means that although more than 20 000 reads (as a total from two samples) were obtained from seawater, only

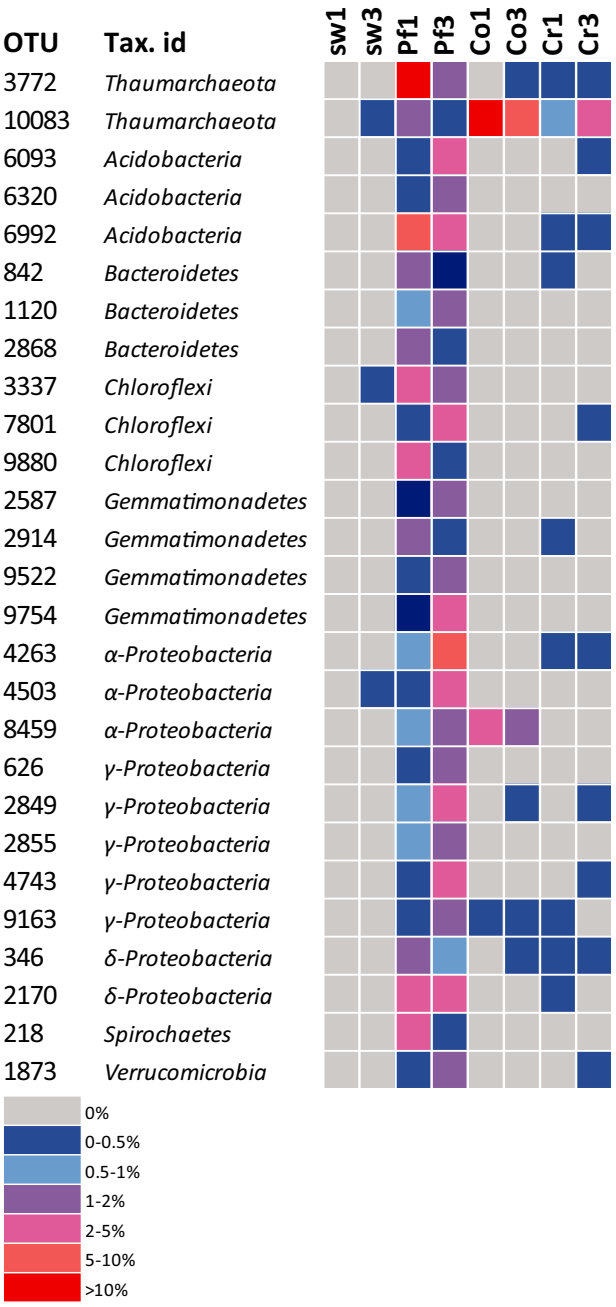



**Fig. 5.** Heat map of the OTUs that represented more than 1% of the reads in at least one of the *P. ficiformis* specimens. Relative abundance of reads belonging to these OTUs is also shown for seawater samples and for *C. candelabrum* and *C. crambe* samples. The complete heat map including all *C. candelabrum*, *C. crambe* and seawater OTUs that represent more than 1% of the reads is presented in Fig. S4.

13 reads (distributed over 3 OTUs) were shared with *P. ficiformis*. It could be foreseen that, according to Baas Becking's hypothesis that 'Everything is everywhere, but the environment selects' (Baas Becking, 1934), also the other *P. ficiformis* OTUs might be discovered in seawater by deeper sequencing or selective amplification. The rarefaction curves of observed OTUs in the seawater samples were not saturated (Table S4), which leaves room for the detection of the 'missing OTUs'. Much lower numbers than 1 in 20 000 may be enriched by pumping sponges that approximately encounter $10^{10}$ bacterial cells per day per ml sponge (Hill, 2004). Presence of these bacteria in the rare biosphere (Pedrós-Alió, 2012) could be sufficient for horizontal transmission. On the other hand, bacteria composition is quite patchy in seawater at both spatial and temporal scales (Eilers *et al.*, 2001), and bacteria found in the sponge tissue at the sampling date could have been acquired by the sponge from seawater with a different microbial composition prior to our sampling of seawater. In that respect, it would be particularly interesting to sample surrounding seawater in the late autumn during spawning and larval development (Maldonado and Riesgo, 2009) to determine whether bacteria that are commonly found in sponges are more prevalent in the seawater during certain times of the year. The massive sequencing efforts during the last decade have revealed that microbes that were previously regarded as sponge specific are actually quite widespread outside the sponge hosts in the rare biosphere of seawater (Webster *et al.*, 2010; Lee *et al.*, 2011; Taylor *et al.*, 2013). The accumulation of sequence data expected in the coming years may further improve our insights in the distribution of bacteria primarily residing in sponges in the environment. A recent paper by Burgsdorf and colleagues (2014) showed that the impact of geography on *P. ficiformis*-associated microbiota was significant, suggesting that for this species, local seawater bacterial communities play an important role in fine-tuning the host microbial community.

Our observations do not shed light on the mechanism of bacterial enrichment in the sponges, and it is unknown whether the sponge recruits its microorganisms, the microorganisms choose their host or if the interplay between host and microorganisms determines the sponge species-specific microbial profile. A study of bacteria associated with the sponge *Cymbastela concentrica* showed that ankyrin repeat and tetratricopeptide repeat proteins that mediate protein–protein interactions in eukaryotes and microorganisms were more than 100-fold overrepresented in the sponge-associated bacterial metagenome when compared with a metagenome of bacterioplankton. These proteins have been proposed to serve the bacteria to evade the sponge's digestive system (Thomas *et al.*, 2010). This implies that the establishment of symbiosis in sponges through horizontal transmission may be mediated by the microorganisms in a 'stealth' manner as proposed for coral-zooxantellae associations (Voolstra *et al.*, 2009). *Petrosia ficiformis* may prove a promising model to study the mechanism of colonization of marine sponges by bacteria upon environmental acquisition, because results are not confounded by vertical

transmission. Difficulties with growing marine sponges in captivity (Sipkema *et al*., 2006) and isolating sponge-specific bacteria (Sipkema *et al*., 2011) currently hamper experimental monitoring and manipulation of *P. ficiformis* by introducing specific bacteria at different developmental stages, but may prove highly instrumental to obtain insights in the mechanism and plasticity of the sponge's associated microorganisms.

## Conclusion

A large proportion of the bacteria present in *P. ficiformis* is closely related to bacteria that have been detected in larvae and embryos of other sponge species. This indicates that similar sponge-associated bacteria can be acquired via both vertical and horizontal transmission.

## Experimental procedures

### *Sample collection*

Five different specimens of each of the sponges *P. ficiformis*, *C. candelabrum* and *C. crambe* were collected by scuba diving at Punta Santa Anna in Blanes, Spain (N41°40′23.46″ E2°48′10.80″) from a 200 $m^2$ rocky area at a depth between 10 and 15 m on 5 June 2008. The samples were immediately transported to the laboratory in excess of seawater and identified by M.J. Uriz based on their morphological and skeletal characteristics. Tissue samples were cut into small pieces (size of a match head), and each sponge piece was separately submerged and rinsed in a large volume of autoclaved artificial seawater (26.52 g NaCl, 2.45 g $MgCl_2$, 0.73 g KCl, 1.14 g $CaCl_2$ and 3.31 g $MgSO_4$ $l^{-1}$) before it was stored in absolute ethanol at −20°C. The ethanol was replaced by fresh ethanol one day after collection. In addition, four samples of 500 ml seawater were collected under water from the same location at the same day.

### *DNA extraction, PCR amplification and DGGE*

Total DNA was extracted from tissue (pieces of the inner core of the sponge were used) of all sponge specimens stored in ethanol according to the tissue extraction protocol from the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) 5 days after collection. Seawater samples were filtered immediately upon collection onto a 0.2 μm polycarbonate filter (GE Osmonics, Minnetonka, MN, USA). The filters were cut into pieces and submerged in lysis buffer (40 mM EDTA, 50 mM Tris, 0.75 M sucrose and pH 8.3) supplemented with lysozyme, proteinase K and sodium dodecyl sulfate. They were extracted using phenol as described previously (Dumestre *et al*., 2002).

Partial 16S rRNA genes were amplified from all samples by PCR using universal bacterial primers bac341F-GC (= 341F with GC-clamp) (CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCC TAC GGG AGG CAG CAG) (Muyzer *et al*., 2004) and bac907RM (CCG TCA ATT CMT TTG AGT TT) (Schauer *et al*., 2000). Polymerase chain reaction amplification was performed in a volume of 100 μl using 1× Taq buffer, 0.2 mM dNTP mixture, 0.8 U Taq DNA polymerase (New England BioLabs, Frankfurt am Main, Germany), 0.5 μM of each primer and 2 μl template DNA. All PCR reactions were done according to the following programme: initial denaturation at 94°C for 5 min; 10 cycles of denaturation at 94°C for 1 min, annealing at 65°C (touchdown to 56°C with 1°C reduction in annealing temperature per cycle) for 1 min and elongation at 72°C for 3 min; 20 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 3 min; and a final extension step at 72°C for 5 min.

Denaturing gradient gel electrophoresis analysis was performed based on the work of Sánchez and colleagues (2007) who tested these primers for Blanes Bay seawater samples. Operating conditions were slightly modified to optimize the results for sponge-derived samples. Briefly, DGGE analysis was performed with a DCode system (Biorad, Hercules, CA, USA). Approximately 900 ng of PCR products of each sample was loaded on a gel with a gradient of 45–75% (100% contained 42 g urea and 40 ml formaldehyde per 100 ml). The gel was run at 100 V for 18 h. Samples were stained with SYBR gold (Invitrogen, Carlsbad, CA, USA). Individual bands were excised and re-amplified by PCR with the same primers (but without GC-clamp) and PCR programme as aforementioned. Polymerase chain reaction products were purified using the Qiaquick PCR purification kit (Qiagen) before they were sent for sequencing (Macrogen, Korea). Sequences were deposited at National Center for Biotechnology Information (NCBI) Genbank under accession numbers GQ258061-GQ258103. Denaturing gradient gel electrophoresis images were further analysed by BIONUMERICS 4.5 software (Applied Maths, Kortrijk, Belgium). Band positions were assigned manually and matched by numerical analysis using the Dice coefficient to calculate similarities between profiles with the unweighted pair–group method using average linkages clustering. Before calculating average similarities within and between sample types artificial double DGGE bands, which were identified based on sequence identity, were removed from the dataset. Intraspecific variation within sample types was calculated using SIMPER in the software package PRIMER 6 v6.1.9 (PRIMER-E Ltd, Plymouth, UK) without using a cut-off for low contributions. One-way ANOSIM was used to test for significant differences between sample types based on binary matrixes using the permutational multivariate analysis of variance (PERMANOVA) extension pack for PRIMER v6.1.9 (PRIMER-E, Plymouth, UK).

### *Sample preparation for pyrosequencing*

Tag-encoded amplicon pyrosequencing was conducted for two *P. ficiformis* (Pf1 and Pf3), two *C. candelabrum* (Co1 and Co3) and two *C. crambe* samples (Cr1 and Cr3). For the seawater samples DNA from sw1 and sw2 was pooled and DNA from sw3 and sw4 was pooled prior to PCR amplification. The primers used were universal 16S rRNA gene primers PRK341F (CCT AYG GGR BGC ASC AG) and PRK806R (GGA CTA CNN GGG TAT CTA AT) to amplify an approximately 466 bp fragment of the bacterial and archaeal 16S rRNA genes comprising the V3 and V4 regions (Youngseob *et al*., 2005). Polymerase chain reaction amplification was performed in a volume of 40 μl using 1× Phusion HF buffer,

2.5 mM magnesium chloride, 0.2 mM dNTP mixture, 0.8 U Phusion Hot Start DNA polymerase (Finnzymes, Espoo, Finland), 0.5 μM of each primer and 1 μl template DNA. Polymerase chain reaction was performed using the following conditions: an initial denaturation at 98°C for 30 s, followed by 30 cycles of denaturation at 98°C for 5 s, annealing at 56°C for 20 s, elongation at 72°C for 20 s and a final elongation at 72°C for 5 min. The samples were checked on a 1.25% (w/v) agarose gel and purified using the Millipore DNA Gel Extraction Kit (Millipore, Billerica, MA, USA). A second round of PCR was performed as described above, except that a pyrosequencing adapter (CCT AYG GRB GCA SCA G) and eight different barcodes of 10 nucleotides length were used with the forward primer. Furthermore, the number of cycles of denaturation, annealing and elongation was reduced to 15. The PCR products were visualized on a 1% (w/v) agarose gel and the bands of PCR products were excised from the gel and purified as described above. The amplified fragments with adapter and tags were quantified using a Qubit fluorometer (Invitrogen) and mixed in approximately equal concentrations ($4 \times 10^5$ copies $\mu l^{-1}$) to ensure equal representation of each sample in the pool. A 454-sequencing run was performed on a GS FLX Standard PicoTiterPlate ($70 \times 75$) using a GS FLX pyrosequencing system according to the manufacturer's instructions (Roche, Mannheim, Germany) at the Technical University of Copenhagen. Pyrosequencing data were deposited at the European Bioinformatics Institute in the sequence read archive under sample accession numbers ERS178680–ERS178687 (http://www.ebi.ac.uk/ena/data/view/ERP001839).

### *Sequence analysis*

Pyrosequencing data were analysed using the QIIME 1.4.0 pipeline (Caporaso *et al*., 2010). Low-quality sequences were removed using default parameters [(i) reads with fewer than 200 or more than 1000 nucleotides, (ii) reads with more than six ambiguous nucleotides, (iii) homopolymer runs exceeding six bases, (iv) reads with missing quality scores and reads with a mean quality score lower than 25 and (v) reads with mismatches in the primer sequence] and OTUs were identified at the 97% identity level. Representative sequences from the OTUs were aligned using PyNAST (DeSantis *et al*., 2006). The taxonomic affiliation of each OTU was determined using the RDP Classifier at a confidence threshold of 80% (Wang *et al*., 2007) against the 12_10 Greengenes core set. Possible chimeric OTUs were identified using QIIME's ChimeraSlayer and removed from the initially generated OTU list, producing a final set of non-chimeric OTUs. The OTU table was used to create an OTU network in QIIME. The generated reduced edge and reduced node tables were exported to Cytoscape 2.8.3 (Smoot *et al*., 2011) to visualize the similarities between microbial profiles of the samples. To cluster the OTUs and samples in this network, we used an unweighted Spring-Embedded algorithm with nodes as physical objects minimizing forces in the network, and edges as springs with a constant and a resting length.

All OTUs representing at least 1% of the reads in one of the eight sponge and water samples were considered for a more thorough phylogenetic analysis. Representative sequences of the OTUs were aligned using the SILVA online SINA alignment service (Pruesse *et al*., 2007). Each OTU was complemented with the two most closely related 16S rRNA gene sequences as determined by a BLAST search against the NCBI nucleotide database (9 September 2011). More neighbours were included if the two nearest neighbours were both sponge or coral derived until a neighbour of non-sponge/coral origin was reached. Neighbour sequences were downloaded from SILVA and together with the aligned OTUs imported in the ARB software package (Ludwig *et al*., 2004). Neighbour sequences > 800 base pairs were used to construct a Bayesian phylogenetic tree. Ambiguous regions of the alignment were systematically removed using the program Gblocks v.0.91b (Castresana, 2000). The default program parameters were used, except allowing a minimum block length of five and gaps in 50% of positions. Phylogenetic trees were created by Bayesian analysis, using MrBayes v3.0b4 (Huelsenbeck and Ronquist, 2001) at the freely available Bioportal server (www.bioportal.uio.no). All parameters were treated as unknown variables with uniform prior-probability densities at the beginning of each run, and their values were estimated from the data during the analysis. All Bayesian analyses were initiated with random starting trees and were run for $10^7$ generations. The number of chains was set to four and Markov chains were sampled every 1000 iterations. Points prior to convergence were determined graphically and discarded. Calculated trees were imported into ARB, and short sequences obtained in this study were subsequently added by use of the ARB parsimony method without changing the tree topologies.

To identify sequence similarity between excised DGGE bands and OTUs from the pyrosequencing, all 16S rRNA gene sequences of DGGE bands were blasted against a database containing the pyrosequencing OTUs using a locally installed version of BLAST2-2-4 (Altschul *et al*., 1990).

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## Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Fig. S1.** Denaturing gradient gel electrophoresis profile of PCR-amplified partial 16S rRNA gene fragments from five *Petrosia ficiformis* (Pf) specimens, four *C. candelabrum* (co) specimens, five *C. crambe* (cr) specimens and four seawater samples (sw). The *P. ficiformis* samples were run on a different gel. They were aligned with the other samples by using reference samples. Numbers placed left of DGGE bands indicate DGGE bands that were successfully excised and amplified. Band number of Pf are in black only to improve their visibility. The observed bacterial richness of especially *C. candelabrum* and *C. crambe* is lower than suggested by the DGGE profile as a number of artifactual double DGGE bands were identified by sequencing of excised and PCR re-amplified bands (e.g. Co22-Co74, Co87-Co88, Co50-Co8, Co116-Co13 and Cr50-Cr65).

**Fig. S2.** Richness of sponge-associated microbial communities. Rarefaction curves are based on OTUs at a 97% sequence similarity threshold, while singletons (OTUs with one read in the total dataset) were excluded.

**Fig. S3.** Bayesian phylogram based on 16S rRNA gene sequences of OTUs representing more than 1% of the reads in *P. ficiformis* and *C. candelabrum* and their nearest neighbours. Operational taxonomic units that include reads from *C. crambe* and seawater are only included if they also represent more than 1% of the reads in *P. ficiformis* or *C. candelabrum*. Operational taxonomic units from this study are in bold and 'Pf' indicates that the OTU contains reads derived from *P. ficiformis* individuals, 'Co' from *C. candelabrum*, 'Cr' from *C. crambe* and 'sw' from seawater. Numbers in parenthesis after the sample type indication refer to the percentage of reads that are included in the corresponding OTU per sample [9381 Pf(0/0.9) means that 0% of the reads of Pf1 and 0.9% of the reads of Pf3 are included in OTU9381]. Yellow boxes indicate vertical–horizontal transmission (VHT) clusters that are supported by posterior probability (PP) values > 95%. These clusters comprise OTUs from both Pf adults and sequences derived from other sponge's larvae or embryos. Grey boxes indicate potential VHT clusters that have PP values < 95%. Numbers above or below the branches correspond to PP values of the Bayesian analysis. Nodes with PP values of < 50 are not indicated.

**Fig. S4.** Heat map of the OTUs that represented more than 1% of the reads in at least one of the *P. ficiformis*, *C. candelabrum*, *C. crambe* or seawater samples.

**Table S1.** Average similarity (%) of DGGE band patterns within groups (*Petrosia ficiformis* = Pf, *Corticium candelabrum* = Co, *Crambe crambe* = Cr, seawater = sw) (in grey) and between groups (in white) based on SIMPER analysis.

**Table S2.** BLASTn result of DGGE band sequences indicated in Fig. S1 against the NCBI nr/nt nucleotide collection (performed at 3 May 2013). Denaturing gradient gel electrophoresis bands in bold are closely related to OTUs present in VHT clusters (number VHT cluster indicated in parentheses). Species names always refer to sponges and corals. The DGGE band sequences were compared with a database containing reference sequences from the OTUs obtained from pyrosequencing using a locally installed version of BLAST2-2-4 at a minimal similarity of 90%. The per cent of identity compared with pyrosequencing OTUs and nearest neighbours is in parentheses behind the OTU/neighbour identifier. Occasional low similarities are caused by low quality of sequenced DGGE bands rather than novelty of the DGGE sequence.

**Table S3.** Per cent similarity matrix of all sequences that are present in VHT clusters (see. Fig. 3A–D). Vertical–horizontal transmission clusters are depicted in yellow and similarity of the *P. ficiformis* (Pf) OTUs with other members of the cluster is depicted in green. When similarity of Pf OTUs within the cluster is lower than 97%, they are indicated in light green. When other similarities within VHT clusters are below 97% they are indicated in orange.

**Table S4.** Species diversity, richness estimates and coverage obtained at genetic distances of 3%.

[a] Singletons have been excluded for counting the number of observed OTUs.

[b] The coverage of richness was estimated by dividing the average observed species by average expected species multiplied by 100.