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Specialized microbiome of the cave-dwelling sponge *Plakina kanaky* (Porifera, Homoscleromorpha)

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Summary

The recent description of the polychromatic sponge *Plakina kanaky* revealed original microsymbionts, with some morphotypes recorded for the first time in Homoscleromorpha, and others never before observed in other sponge groups. Illumina 16S amplicon sequencing was used to characterize this microbial community by comparing contents of seven specimens of this Plakinidae with five other sponge species: 1 Homoscleromorpha of the Oscarellidae family and 4 Demospongiae. A total of 256,458 sequences of the hypervariable V5-V6 region of the 16S rRNA gene were clustered into 2,829 OTUs at 97% similarity, with Proteobacteria, Poribacteria and Chloroflexi being the most abundant phyla. The *Plakina kanaky* specific community appeared to be mainly composed by 5 OTUs representing about 10% of the total microbiome. Among these, the filamentous bacterium *Candidatus Entotheonella*, which was among the dominant morphotypes previously observed in the mesohyl and the larvae of *P. kanaky*, was detected in all studied specimens. However, other original and dominant morphotypes could not be assigned to a known prokaryotic taxon. This cave dwelling sponge species harbors a distinctive microbiome composition of potential taxonomic and metabolic novelties that may be linked to its ecological success in such extreme environments.

Keywords: Microbiome, *Entotheonella* sp, vertical transmission, species-specific community

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Introduction

Sponges (Phylum Porifera) are well known marine sessile organisms living in close association with symbiotic Bacteria, Archaea and unicellular Eukaryotes. The microbial communities of these holobionts are often complex in terms of diversity and biotic interactions (Thomas *et al.* 2016). Transmission electron microscopy has allowed the description of a great variety of microbial morphotypes that can be found in the sponge mesohyl, inside specialized cells and even inside their nuclei. In some sponge species, known as bacteriosponges or High Microbial Abundance (HMA) sponges, the microbial part of the holobiont can constitute up to 40% of the total biomass represented by a diverse microbial community (Vacelet, 1975; Webster and Taylor 2011; Hardoim *et al.* 2012; Uriz *et al.* 2012). It is well accepted that sponge-associated communities are therefore a significant source of unique microbial diversity (Hentschel *et al.* 2002; Taylor *et al.* 2007; Thomas *et al.* 2010; Reveillaud *et al.* 2014). Sponges harbor rather species-specific prokaryotic communities, composed both by generalist and specialist microbes (Easson and Thacker 2014; Thomas *et al.* 2016). The rate of host specificity depends on the modes of symbiont transmission, which can be horizontal (from the environment), vertical (through reproduction) or a mixture of both (for a review see Thacker and Freeman 2012). To identify processes of vertical transmission, the symbionts transmitted from parents to offspring can be distinguished using microscopic techniques or traced using molecular tools within the larvae. These associations are often assumed to represent the composite result of co-evolutionary processes, which thus shape the microbiome diversity, influenced by the host specificity and the network of metabolic interactions within the holobiont (Thomas *et al.* 2010; Pita *et al.* 2018).

Recent advances in next generation sequencing technologies have led to important progress in the study of microorganisms in their natural environments, surpassing some of the limitations of culture-based methodologies, and revealing new insights into sponge symbiotic diversity and functions (Schmitt *et al.* 2011; Hentschel *et al.* 2012). For instance, the retrieval of 16S rRNA gene sequences from metagenomic DNA revealed 52 microbial phyla associated with sponges (Webster and Thomas, 2016), the most common of which are Proteobacteria, Actinobacteria, Chloroflexi, Bacteroidetes, Acidobacteria, Cyanobacteria, Firmicutes and the candidate phylum Poribacteria. The Archaea lineages Crenarchaeota and Euryarchaeota are also well represented (Fieseler *et al.* 2004; Taylor *et al.* 2007; Webster *et al.* 2010; Thomas *et al.* 2016).

Among Porifera, Homoscleromorpha is the smallest class, currently composed of 124 valid species distributed within two families, Oscarellidae and Plakinidae, and nine genera (Van Soest *et al.* 2020). Sponges of this group often inhabit semi-dark or dark habitats such as overhangs or submarine caves in shallow waters. The current knowledge of the symbiotic microorganisms of this group is mostly based on electron microscopy observations, with molecular studies enabling a better assessment of the microbial diversity remaining scarce (Gloeckner *et al.* 2013). The most recent cytological descriptions of various species have revealed dense microbial communities in which the dominant prokaryotic morphotypes are often used as diagnostic traits for the challenging taxonomy of this sponge group. Oscarellidae are mostly LMA sponges, while Plakinidae are generally HMA sponges (Boury-Esnault, Muricy and Gallissian 1995; Muricy *et al.* 1996, 1999; Gloeckner *et al.* 2013; Pérez and Ruiz 2018).

Plakina kanaky (Ruiz *et al.* 2015) is an HMA polychromatic Homoscleromorpha sponge recently described in New Caledonia and harboring an original prokaryotic community, containing, among other morphotypes, a globulous shape never before observed in any Porifera, as well as a filamentous morphology recorded for the first time in

Homoscleromorpha (Ruiz *et al.* 2015). TEM observations indicated a common microbial symbiotic community among the various specimens collected, with only subtle variations suspected to be related to sponge chromotypes. The vertical transmission of the two main morphotypes has also been proposed after their observation inside the coeloblastula larva of *P. kanaky* (see Ruiz *et al.* 2015 and Fig 1). This study aims to characterize the prokaryote community associated with *P. kanaky*, combining the previous morphological description with molecular analyses of the 16S rRNA gene amplicon sequencing in order to: 1) define the patterns of ribotypes detected in *P. kanaky* individuals compared to other reported sponge microbiomes in the present study; 2) determine if part of the microbial community of *P. kanaky* is species-specific as a first step towards the identification of the original morphotypes previously observed; and 3) define the putative mode of transmission of the microbial taxa representatives deduced from Operational Taxonomic Units (OTUs) and their reported lifestyles.

[FIG 1]

Material and Methods

Sponge collection and preservation

Seven specimens of *P. kanaky* were collected in November 2010 and February 2013 by scuba diving in New Caledonia (Tropical Southwestern Pacific Ocean), at Bourail (21°24.303' S, 165°27.811' E), Hienghène (20°36.756' S, 164°56.958' E), and Touho (20°45.503' S, 165°16.964' E), in small dark cavities located between 15 and 25 m depth along the outer slope of the reef (Table 1). These samples were also used for the description of the new species (Ruiz *et al.* 2015). For molecular analyses, specimens were preserved in ethanol 95%. For comparative and outgroup purposes, we determined the microbial composition using the same approach of four sponge species (one specimen per species): *Amphimedon compressa*, *Aplysina cavernicola*, *Petrosia ficiformis* (Demospongiae) and *Oscarella balibalo* (Homoscleromorpha).

[TABLE 1]

Metagenomic DNA extraction, 16S rRNA gene library construction and sequencing

Total DNA extraction from a small (2 cm³) fragment of sponge was performed using a FastDNA™ SPIN KIT (MP Biomedicals). PCR amplicons of the hypervariable V5-V6 region of the 16S rRNA genes were obtained from sponge metagenomic DNA as a template using the primers 807F [5'-GGATTAGATACCCBRGTAGTC- 3'] and 1050R [5'-AGYTGDCGACRRCCRTGCA-3'] (Bohorquez *et al.* 2012). The PCR cycle conditions and Illumina library preparations were performed as previously described ((Milici *et al.* 2016; Villegas-Plazas *et al.* 2019) .

Bioinformatics analysis

All sequence analyses were performed using QIIME 1.9.0 (Kuczynski *et al.* 2012) and the Phyloseq R package 1.5.21 (McMurdie and Holmes 2013). A set of Illumina reads was initially filtered and split according to the barcodes. Chimeric sequences were identified and excluded from the datasets using Usearch 6.1. A multi-step open-reference OTU picking workflow was performed within the QIIME system. OTUs were picked, assigning the reads to species groups based on 97% sequence similarity. All analyses and multivariate tests were performed using the OTUs relative abundances of the detected microbial community compositions on each sample. Each OTU with a relative abundance higher than 1% was considered as a key microbial community component of a given sample, and

among these, the species-specific microbial community composition was defined as the set of OTUs present in all specimens of *P. kanaky*.

Alpha and Beta diversity analyses were performed using QIIME workflow and R software using Phyloseq (McMurdie and Holmes 2013) and Vegan R packages (Oksanen *et al.* 2019). All bar plots of abundance, diversity and distribution of all phyla were prepared with the Phyloseq R package. Weighted Unifrac distances were calculated between the microbial communities of the sponge specimens based on OTU presence and relative abundance (Lozupone *et al.* 2011). The same distance method was used for Beta diversity using the function *Adonis* using R software.

A heatmap based on a Discriminatory Analysis of Partial Least Square Regression PLS-DA analysis was constructed using the online platform MetaboAnalyst (Xia and Wishart, 2016). We compared the 50 most important OTUs (Variables Important in Projection, VIP) on the basis of their occurrence in terms of number of reads distributed among all *P. kanaky* samples.

Results

A total of 256,458 sequences of 250 nucleotides on average were obtained and clustered into 2,829 OTUs at 97% similarity (Table 1). Rarefaction analyses showed that all *P. kanaky* samples similarly approached an asymptote near 6,000 sequence reads retrieved per amplicon describing more than 96% of the associated microbial communities, according to Good's coverage estimation (Supp Fig. 1). Beta diversity analysis using Permanova test revealed that the microbial community in *P. kanaky* presents no significant differences among sites ($F=0.83$; $p\text{-value}=0.74$) and chromatotypes ($F=0.78$; $p\text{-value}=0.76$). The *P. kanaky* microbial community is composed of 14 formerly described Bacteria phyla and 1 Archaea lineage. Proteobacteria, Poribacteria and Chloroflexi are the predominant taxa constituting up to 70% of the bacterial community in all samples (Fig. 2A). At class level, 80% of OTUs were correctly assigned, however at the order level we found that 40% of the sequences could not be assigned to a known taxon.

Four abundant microbial classes represent between 60 and 80% of the OTUs recorded in the *P. kanaky* microbiome. They belong to the Deltaproteobacteria (12 – 40%), to an unclassified class of Poribacteria (12 – 38%), to the SAR202 cluster (7 – 22%), and to the Gammaproteobacteria (4 – 10%) (Fig. 2A-B). At the order level, only the order Entothaeonellales (Deltaproteobacteria) could be identified among the 4 dominant classes recorded in the *P. kanaky* microbiome. However, other less abundant orders could be identified. They belong to the Acidimicrobiales (Class Acidimicrobia), 2-7% of the OTUs recorded, and to the Caldilineales (Class Anaerolineae) with 0.5-5% of the total microbial community. Archaea also represents less than 2% on average of the total prokaryotic community associated with *P. kanaky*, and they are represented by two families of the class Thaumarchaeota: Nitrososphaeraceae and Cenarchaeaceae.

[FIG 2]

We performed intraspecific and interspecific comparisons of the microbial communities by generating and including in our datasets the microbiomes of another Homoscleromorpha and of three Demospongiae. This enabled us to compare the ribotypes detected and to characterize the species-specific community associated with *P. kanaky* specimens. Compared to other sponges analyzed, the largest difference (40%) was found between all *P. kanaky* specimens and *Amphimedon compressa*, while the community associated with the Homoscleromorph *Oscarella balibalo* appeared as the most similar with less than 20% divergence between both microbial communities. Two Chloroflexi OTUs were found in all sponge samples, one affiliated to the SAR202 cluster and the other one to the class TK17.

The greatest divergences were observed when the abundance was not considered (unweighted). In this case, two groups corresponding to the Homoscleromorpha and Demospongiae were well separated (Fig. 2B). When the presence/absence of OTUs among samples were compared, such unweighted measures suggested that the microbial divergence between Homoscleromorpha samples is about 50%, whereas it reaches 70% with the Demospongiae *Amphimedon compressa*, whose microbiome is mainly composed by Betaproteobacteria of the EC94 order (89% of the OTUs). Since *A. compressa* is an HMA sponge species, it represents an exceptional contrasting pattern where phyla proposed to be LMA (Betaproteobacteria) and HMA (SAR202) indicators are coincidentally having a higher relative abundance. Searching for the common features of both Homoscleromorpha, we were able to detect some of the dominant OTUs of *P. kanaky* in *O. balibalo*, one belonging to Poribacteria and two Chloroflexi: the SAR202 cluster and the Caldilineales order. Taken together, these three taxonomic groups, which are shared by both species, represent half of the total microbial community of the Oscarellidae.

The species-specific community of *P. kanaky* appears to be composed of 5 bacterial OTUs recorded in all studied specimens and representing about 10% of the total microbial community. These specific OTUs belong to Acidimicrobiales, an order in the phylum Actinobacteria, to two different SAR202 cluster in the Chloroflexi phylum, to the order Sva0725 in the Acidobacteria phylum, and to the family Endozoicomonadaceae in the Gammaproteobacteria class. Also observed in all *P. kanaky* samples, the Archaea genus *Nitrosopumilus* (Cenarchaeaceae) can be considered as a minor part of the species-specific community.

To complete our view of the species-specific community of *P. kanaky*, we created a heatmap showing the distribution of the 50 VIP OTUs among the various *P. kanaky* chromatotypes. This representation indicates that 6 OTUs are shared by at least 6 *P. kanaky* specimens. They were assigned to *Candidatus solibacter*, to the family Methylococcaceae, to SAR202, HTCC2089 (Gammaproteobacteria) and to *Candidatus Entotheonella*. This candidate genus is actually represented by 6 different OTUs heterogeneously distributed in all *P. kanaky* specimens (Fig. 3), but because of the genetic differences between *Entotheonella* OTUs (divergences greater than 3%), this taxon was not originally detected as a part of the species-specific microbial community. However, even if not a single *Entotheonella* OTU could be detected in all specimens of *P. kanaky*, our previous electron microscopy observations of such morphotypes in all specimens and their larvae convinced us to assume this taxon as being part of the species-specific microbial community.

[FIG 3]

Discussion

The availability of high-throughput meta-barcoding approaches allows more accurate descriptions of the microbiomes associated with marine sponges. So far, only a few Homoscleromorpha have been investigated using such methods, although cytological observations of microbial morphotypes are often used as diagnostic traits to distinguish between species (Ereskovsky, Sanamyan and Vishnyakov 2009; Pérez *et al.* 2011; Ruiz *et al.* 2017). Previous work on the HMA *Plakortis* sp. has highlighted the occurrence of 8 bacterial phyla, among which the Chloroflexi and Acidobacteria that contain many sponge-specific clusters (Taylor *et al.* 2007). Sharp *et al.* (2007) studied the vertical transmission of some original phylotypes of Proteobacteria, Actinobacteria, Nitrospira and Chloroflexi in *Corticium* sp.; among these phyla, the closest previously known bacterial relatives were sulfur-oxidizing Gammaproteobacteria, nitrogen-fixing *Rhizobiales* and Actinobacteria, with the latter phylum being recognized for its ability to produce bioactive compounds (Sharp *et al.* 2007).

Most of the species-specific community associated with *P. kanaky* can be classified among Proteobacteria and Poribacteria, but Actinobacteria and Chloroflexi are also recorded in abundance. Proteobacteria is mostly represented by Delta- Gamma- and Alphaproteobacteria. Four orders dominate, Enttheonellales (Deltaproteobacteria with 11 – 38 % of relative abundance), an unidentified Poribacteria (12 – 38%), a second unidentified order belonging to the class SAR202 (Chloroflexi), and Acidimicrobiales (Actinobacteria with 3 – 7 % of relative abundance).

Poribacteria have been associated with polyketides and methyl-branched fatty acid production (Hochmuth *et al.* 2010), suggesting also their involvement in the production of secondary metabolites. Three OTUs are related to the Chloroflexi class SAR202 and account for half of the total specific community. SAR202 has also a good number of representatives which have been recorded in soil, freshwater, marine, wastewater, and sub-surface environments (Morris *et al.* 2004). While the global bacterial abundance generally decreases with ocean depth, the absolute abundance of SAR202 increases from the sub-surface to the meso- and bathypelagic waters (Varela *et al.* 2008). This distribution pattern indicates that SAR202 is well adapted to the environmental conditions of the dark ocean, such as the small dark cavities where *P. kanaky* dwells. Using MICRO-FISH, Varela *et al.* (2008) found that uptake of L-Asp by SAR202 cells remains stable throughout the water column, suggesting that this cluster may play a key role in lower-surface and deep-ocean biogeochemistry. The order Acidimicrobiales contains mesophilic and moderate thermophilic species, of which all members are obligatory acidophilic and are found in iron-, sulfur- or mineral-sulfide rich environments. Species within this order are capable of ferrous iron and sulfur oxidation and ferric iron reduction (Clark and Norris 1996; Stackebrandt, Rainey and Ward-Rainey 1997; Johnson *et al.* 2009). Taylor *et al.* (2007) found several sponge-specific clusters in tropical sponges such as *Agelas dilatata*, *Discodermia dissoluta*, *Plakortis* sp. and *Xestospongia muta* from the Caribbean Sea, *X. testudinaria* from Indonesia and *Dysidea* sp. from the Southern China Sea.

Enttheonella sp., first identified in Tetractinellid sponges (Schmidt *et al.* 2000; Schirmer *et al.* 2005; Brück *et al.* 2008), is a bacterial group known for being a unique source of bioactive molecules (Bewley, Holland and Faulkner 1996; Jaspars and Challis 2014; Wilson *et al.* 2014). Widely distributed throughout the ocean, this phylotype is generally considered to be a free-living planktonic bacterium, acquired by sponges from the environment (Wilson *et al.* 2014). However, the cytological description of *P. kanaky* reported the predominance of a filamentous *Enttheonella*-like bacteria in all four chromatotypes of *P. kanaky* as well as in their embryos (Ruiz *et al.* 2015). The present study confirms the occurrence of *Enttheonella*, and the great novelty of this first record in Homoscleromorph sponges also provides the first clues to its vertical transmission. In the species-specific community of *P. kanaky*, *Enttheonella* is represented by 12 different OTUs with 97% similarity. However, we believe that small genetic divergences in the hypervariable V5 – V6 region may explain the assignment of various OTUs to *Candidatus Enttheonella* sp. Considering the large specialized biosynthetic repertoire reported for *Enttheonella*, its close association to *P. kanaky* likely confers great properties of adaptation to the whole sponge holobiont. Finally, regarding archaeal groups detected in *P. kanaky*, they had been reported with steady detection and low abundances in the microbiomes of most sponge species surveyed so far (Thomas *et al.* 2016) with only few reported exceptions where these groups are predominant (Turque *et al.* 2010; Jackson *et al.* 2013; Garcia-Bonilla *et al.* 2019). Among them, the occurring OTUs assigned to *Thaumarchaeota* are associated with genera playing essential metabolic activities such as ammonia oxidation, which is one of the first reactions carried out to achieve nitrification (Steger *et al.* 2008). Members related to the archaeal clade of the *Nitrososphaera*-

Nitrosopumilus cluster (Stieglmeier *et al.* 2014) could be the globulous prokaryotic form described in *P. kanaky* adults and larvae (Ruiz *et al.* 2015), a morphotype never before observed in sponges. This is a possibility still to be confirmed and requiring further investigation. It must be noted that the use of more specific molecular markers might reveal a much higher diversity of symbiotic Archaea in sponges, particularly in those living in extreme environments, and might thus highlight other putative adaptive capabilities.

The microbial community associated to *Plakina kanaky* has no significant differences among chromotypes and/or sites. It is likely that this community is transferred from parents to offspring, while other OTUs can be acquired from the environment. Both types of acquisition are key processes which may improve the adaptive capabilities of a holobiont living in constraining environments such as underwater caves. The microbial diversity associated with Homoscleromorpha may thus partly explain the ecological success of this poorly known sponge group in dark and oligotrophic environments. This preliminary study may thus serve as a baseline for further research on the adaptative capabilities of Homoscleromorpha.

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Figures

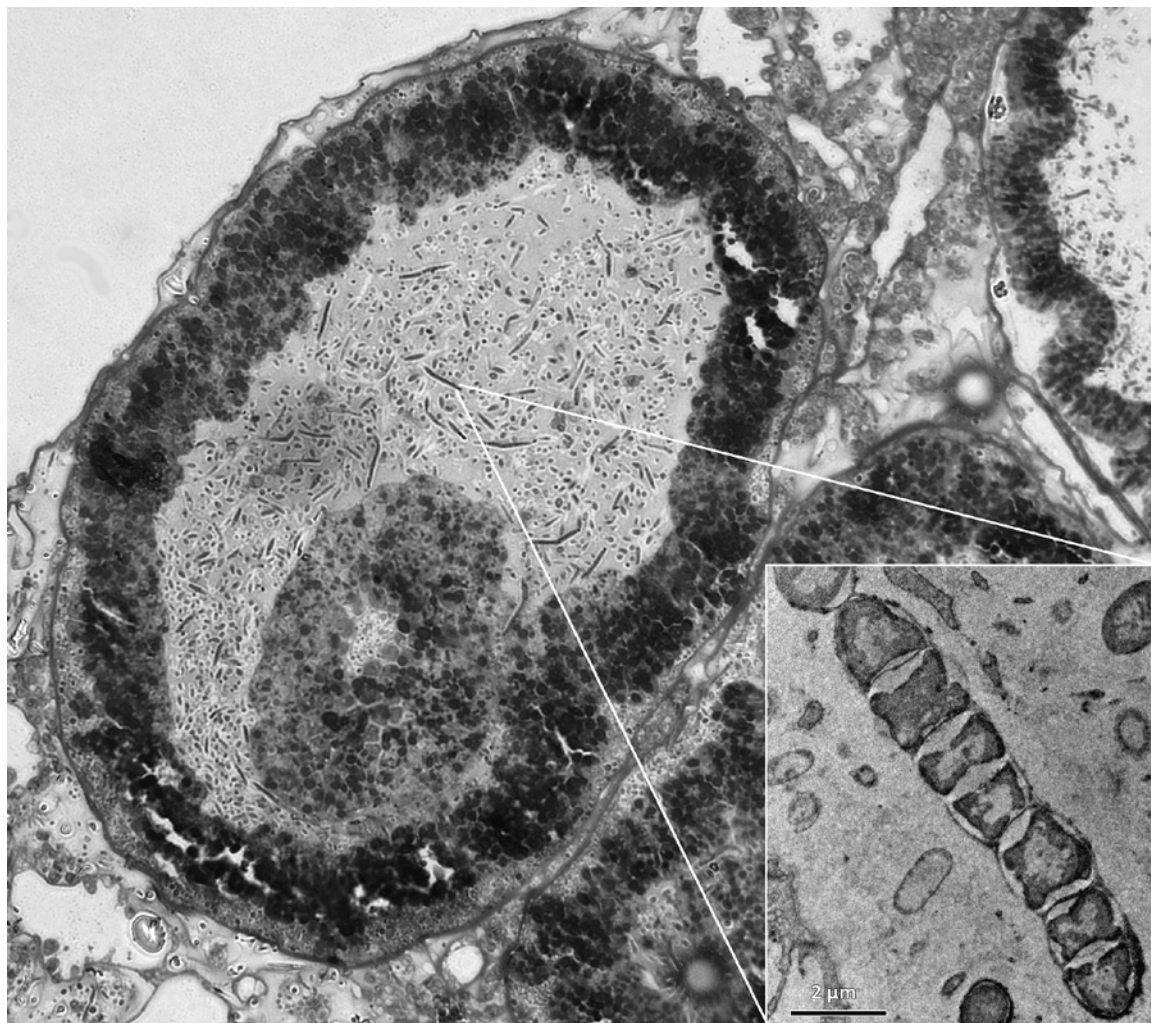


Figure 1. Coeloblastula of *Plakina kanaky* with a detailed view of the filamentous morphotype associated with *Candidatus Entotheonella* sp., found in both the adult and the larvae of *P. kanaky*.

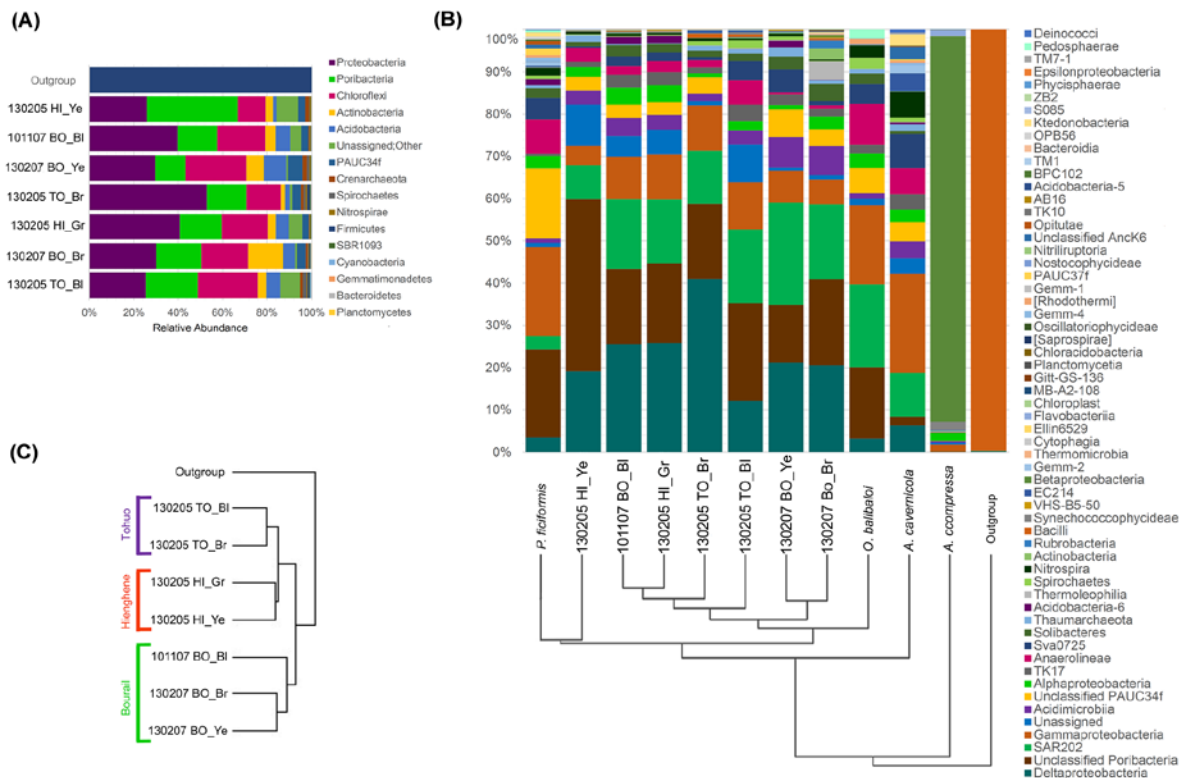


Figure 2. (A) Occurrence of all phyla inferred from 16S amplicon Illumina reads with an average of 8504 sequences per specimen. (B) Relative class abundance inferred from 16S amplicon Illumina reads with an average of 9820 sequences per specimen and a hierarchical clustering through a weighted UniFrac matrix. (C) Similarity of the samples throughout the Bray Curtis method according to the number of shared OTUs.

Plakina kanaky specimen's abbreviations: TO, Touho. BO, Bourail. HE, Hienghène. BI, bleu. Br, Brick-Red. Ye, Yellow. Gr, Green.

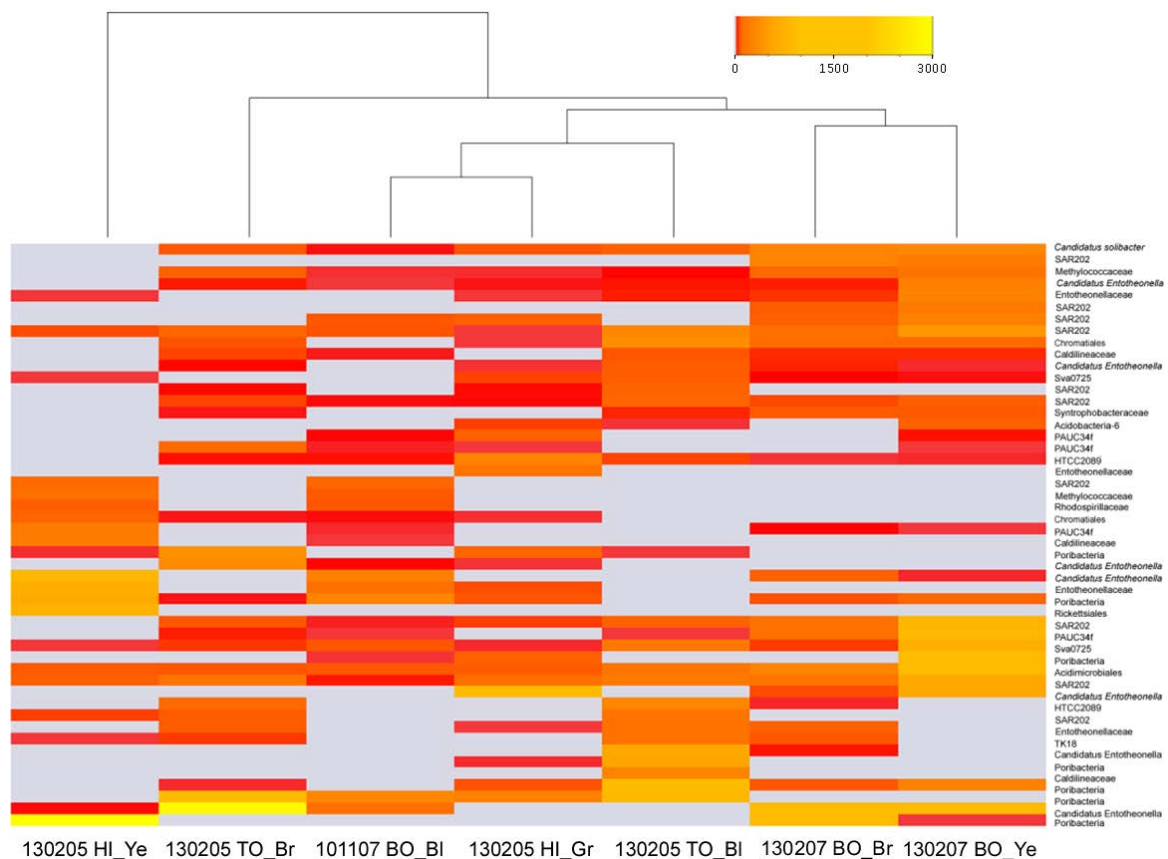


Figure 3. Heatmap comparing the 50 most abundant OTUs in *Plakina kanaky* chromotypes, and dendrogram indicating the intra- and interspecies relationships (top). Heat colours indicate number of reads per taxa, from zero reads (grey) to 3000 reads (yellow). Only the OTUs which are shared by at least 6 chromotypes are marked with black circles and listed in order of appearance (from top to bottom) on the heatmap. Red circles indicate the OTUs assigned to *Candidatus Entotheonella*.

Plakina kanaky specimen's abbreviations: TO, Touho. BO, Bourail. HE, Hienghène. Bl, bleu. Br, Brick-Red. Ye, Yellow. Gr, Green.

Table 1. Individuals and collection sites of the studied specimens (11) with the number of reads and OTUs (97 % similarity) per specimen. NC: New Caledonia; FR: France.

<i>Sponge species</i>	<i>sample code</i>	Chromo type	Collection site	# Reads	# OTUs
<i>Plakina kanaky</i>	130207-BO_Ye	Yellow (Ye)	Bourail (BO)	17024	852
<i>P. kanaky</i>	130207BO_Br	Brick-Red (Br)	Bourail (BO)	12913	915
<i>P. kanaky</i>	101107BO_Bl	Blue (Bl)	Bourail (BO)	5818	376
<i>P. kanaky</i>	130205TO_Br	Brick-Red (Br)	Touho (TO)	10470	520
<i>P. kanaky</i>	130205TO_Bl	Blue (Bl)	Touho (TO)	12713	556
<i>P. kanaky</i>	130205HI_Ye	Yellow (Ye)	Hienghène (HI)	13351	413
<i>P. kanaky</i>	130205HI_Gr	Green (Gr)	Hienghène (HI)	7201	409
<i>Amphimedon compressa</i>	ACM.C3	NA	La Martinique	13203	298
<i>Aplysina cavernicola</i>	FA.M5	NA	Marseille (FR)	38489	919
<i>Petrosia ficiformis</i>	PF.M5	NA	Marseille (FR)	35031	737
<i>Oscarella balibalo</i>	OB.M7	NA	Marseille (FR)	24395	440

Supporting Infomation

Supplementary figure 1. (A) Chao1 indicator. This plot is showing the coverage of sequencing and the richness of all samples. (B) Rank abundance plot of OTUs and sequence accumulation on each specimen observed at 97% similarity in datasets. *Plakina kanaky* specimen's abbreviations: TO, Touho. BO, Bourail. HE, Hienghène. Bl, bleu. Br, Brick-Red. Ye, Yellow. Gr, Green.