Environmental heterogeneity and microbial inheritance influence sponge-associated bacterial composition of *Spongia lamella*

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ABSTRACT

Sponges are important components of marine benthic communities. High-Microbial-Abundance sponges host a large diversity of associated microbial assemblages. However, the dynamics of such assemblages are still poorly known. In this study, we investigated whether bacterial assemblages present in *Spongia lamella* remained constant or changed as a function of the environment and life cycle. Sponges were collected in multiple locations and at different times of the year in the western Mediterranean Sea and in nearby Atlantic Ocean to cover heterogeneous environmental variability. Co-occurring adult sponges and offsprings were compared at two of the sites. To explore the composition and abundance of the main bacteria present in the sponge mesohyl, embryos, and larvae we applied both 16S rRNA gene-Denaturing Gradient Gel Electrophoresis (DGGE) and sequencing of excised DGGE bands, and quantitative Polymerase Chain Reactions (qPCR). On average, the overall core bacterial assemblage showed over 60% similarity. The associated bacterial assemblages fingerprints varied both within and between sponge populations, and the abundance of specific bacterial taxa assessed by qPCR significantly differed among sponge populations and between adult sponge and offsprings. Sequences showed between 92 to 100% identity to sequences previously reported in Genbank, and all were affiliated with uncultured invertebrate bacterial symbionts (mainly sponges). Sequences were mainly related to *Chloroflexi* and *Acidobacteria*, and a few to *Actinobacteria* and *Bacteroidetes*. Additional populations may have been present under detection limits. Overall, these results support that both ecological and biological sponge features may shape the composition of endobiont bacterial communities in *S. lamella*.

INTRODUCTION

A large proportion of marine organisms is hidden and yet undescribed, either as cryptic species [1] or as symbionts [2,3]. A non-negligible part of the disregarded bacterial communities is hidden both inside and on the surface of marine invertebrates, living as associated or symbiotic partners [4-8]**.** That is particularly true for many marine sponges. Microorganisms of the three domains of life [9-11] constitute up to 40% of the sponge biomass [6, 12]. Bacteria seem to benefit from the stable and fertile environment provided within sponge tissues (e.g., food supply, and specific ecological conditions including protection against both UV and potential predators), and the composition of endobiont bacterial communities would be shaped by ecological and biological sponge features, such as environmental factors, habitat, health status, and life cycle among others.

The factors that structure sponge-bacteria associations remain however poorly understood. It is now commonly admitted that sponge associated bacteria are both distinct from the free-living marine bacterioplankton [11, 13-15], and sponge-specific [16] or even species-specific [17]. Although some symbionts communities seemed first-sighted to form stable associations across spatial and temporal scales [5, 16, 18], other studies have shown that microbial communities can be affected by environmental changes: geographical variation [13, 19], metal pollution [20], transfer into aquaculture [21, 22], or by physiological changes such as disease outbreak [23]. Furthermore, host-specific factors are also believed to play a strong structuring force shaping microbial communities as different sponge species from the same habitat harbored distinct communities [24], and the same sponge species from the different habitat showed similar communities [25]. Vertical transmission has been observed in various marine sponges [7, 15, 26-29] and may also strongly shape symbionts composition explaining the occurrence of sponge-specific bacterial clades in diverse hosts and locations [11].

Regardless the mechanisms of bacterial transmission and acquisition, it is unclear whether horizontally or vertically transmitted symbionts are equally relevant or whether they represent the totality or a subsample of the microbes found in adult tissues. Comparing effects on bacterial symbionts of both environment and host have not been performed yet but would unravel factors that structure sponge microbial communities. Moreover, most of the studies were carried out with a snapshot sampling strategy with limited replication [30, 31]. Limited sampling design may hinder conclusions on the variability of sponge bacterial diversity since it fails to properly address the ecological variation in sponge-associated microorganisms.

Populations of *S. lamella* (previously known as the Mediterranean *S. agaricina*, [32**]**) are scattered throughout the Atlanto-Mediterranean region (e.g., [33**])**, where this species has traditionally been collected as a bath sponge [32**]**. Although bacterial assemblages have been reported as inhabitants of the genus *Spongia* [34**]**, we know little about the microbial consortium of *S. lamella* [35**]**. In the present study, we investigated bacterial symbionts from sponges submitted to heterogeneous environmental conditions and collected in multiple locations and at different times of the year in the western Mediterranean Sea and in nearby Atlantic Ocean. Assuming that similarity between adults and offspring bacterial communities is mainly consequence of vertical transmission, we also used a quantitative approach to examine the main bacteria in adults, embryos, and larvae. This part of the study aimed at gaining insight on the transfer and the effect of microbial inheritance on symbionts communities, *i.e.*, whether or not bacteria are randomly transferred to the next generation, and whether or not embryos or larvae harbor the same bacterial communities than their parents. Characterizing this variability is a critical step to unravel the distribution of associated symbionts, sponge-bacteria interactions, and species-specific factor that shape symbionts communities.

MATERIALS AND METHODS

Sampling. *Spongia lamella* (Demospongiae, Dictyoceratida, Spongiidae) individuals were collected by SCUBA diving in nine locations (Fig. 1) along the North West Mediterranean and the Atlantic (see more details in [36]). In the area of Marseille (France), specimens were gathered in Pharillon (43°12'N, 05°20'E), Plane (43°11'N, 05°23'E) and La Ciotat (43°9'N, 05°35'E). In Catalonia (Spain), specimens were collected in Arenys (41°35'N, 02°33'E), and in Cap de Creus (42°17'N, 3°18'E). Populations were also collected in Cabrera (39°7'N, 02°57'E; Balearic Islands, Spain), Ceuta (35°53'N, 05°18'W; Gibraltar area, Spain) and in the Atlantic alongside Portugal (39°26'N, 9°30'W; Berlengas archipelago). Specimens were collected along three years (2005-2007). When it was possible 10 specimens were collected at each location. Three sponge specimens collected in Marseille in 2006 were selected because of the presence of embryos, which gathered in clumps in brooding chambers into the parent mesohyl (Fig. 2A), and were manually dissected. Visually, density of embryos varied between individual sponges, however as no measurement was made, we could not determine any correlation with sponge size. Three additional samples containing mature larvae were collected from Els Bullents (Cala Canyelles, Lloret de Mar; 41º 42' N, 02º 52' E) in July 2008. About 30 minutes after the transfer of the 3 sponges from Els Bullents into individual aquaria, we observed the release of larvae from each of the tree sponges. Larvae of *S. lamella* were oval and showed a black pigmented ring located at one of the larval poles, surrounded by flagella which protrude outside. The other larval pole displayed a hummock without any particular pigmentation (Fig. 2B).

Sponge tissues were transferred in plastic bags containing seawater and stored in a cooler with ice until further processing (usually 2h after sampling). Samples were then rinsed and kept in a series of absolute ethanol baths to prevent ethanol dilution by sponge water content and sponge tissues modification. Sponge tissues were finally preserved in absolute ethanol (100 % final concentration) and stored at –20°C until processed.

Extraction and amplification of bacterial DNA. DNA was extracted with the methodology previously described [37] from sponge mesohyl tissue (around 4 mm^3), embryos (a pool of embryos from the same brooding chamber), and larvae (individually). DNA extracts were dissolved in 50 µl of sterile autoclaved water and run in an agarose gel to check integrity and concentration using a standard mass ladder (DNA Ladder, Bioron). Very small differences in yield extraction were visualized among samples. Such differences were not expected to produce qualitative changes in the DNA mixtures, and the results were normalized using relative abundances in the fingerprinting analysis for an accurate inter-samples comparison. DNA extracts were kept at –20°C until use.

A fragment (c. 600 bp) of the bacterial 16S rRNA gene was PCR amplified with universal bacterial primers 341F (5'-CCT ACG GGA GGC AGC AG-3') with a GC-clamp at the 5'-end and 907RM (5'-CCG TCA ATT CMT TTG AGT TT-3') [38]. The 50 µl PCR mixture consisted of 80 ng of genomic DNA, 5µl of 10X Taq polymerase buffer, 0.75 µl of $MgCl_2$ (100µM), 1 µl of dNTP (10 mM each), 2.5 µl of each primers (10µM), 1.5µl of Bovine Serum Albumin (BSA at 6mg/ml), and 1.25 U Taq polymerase (Boiron). Sigma pure Water was used as negative control. Touchdown PCR was performed in a MWG Primus thermocycler. The entire amplification program was 5 min at 94°C, 10 cycles of 1 min at 94°C, 1 min at 71°C (with 1°C decrease every cycle), 3 min at 72°C, 20 cycles of 1 min at 94°C, 1 min at 61°C, 3 min at 72°C and a final extension time of 3 min at 72°C. Then PCR

products were run in 1.5% agarose gels, stained in an aqueous ethidium bromide solution, and visualized under UV next to a standard mass ladder (DNA Ladder, Boiron).

DGGE and 16S rRNA gene sequencing. DGGE analysis was run in a Bio-Rad Dcode universal Mutation Detection System (BioRad) on 6% polyacrylamide gel in 1X TAE (40 mM Tris Base, 20 mM Sodium acetate trihidrate and 1 mM EDTA). We used a 40-75 % vertical denaturant gradient (100 % denaturant agent is 7 M urea and 40 % deionized formamide). Comparable amounts of PCR products (c. 600 ng DNA) were loaded for each sample as reported [38]. Gels were run for 5 hours at 195 V at 60°C and stained with nucleic acid stain SybrGold (Molecular Probes) solution (0.125 µl/ml) for 45 min, rinsed with MilliQ water and photographed with Geldoc system (supplementary Fig.S1). Images of the gels were analyzed using the Gels plot lanes tool of ImageJ software 1.38X (Wayne Rasband, National Institutes of Health, USA). After background subtraction, the intensity of each band was measured by integrating the area under the peak and was expressed as percentage of the total intensity in the lane. This allowed measurement of the relative abundance of each band and comparison of bacterial fingerprints among and within sponge populations. Bands with intensities <1% of total intensity were excluded.

Prominent bands were excised from the gel under UV, resuspended in 25 µl of MilliQ water and stored at 4ºC overnight. An aliquot (1-4 µl) of supernatant was used for PCR reamplification with the original primer set, and the PCR product was sequenced using external sequencing facilities (www.macrogen.com). Sequences were sent to BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) to get a first indication of the sequence affiliations, and to determine their closest relative in the database. Sequences were also inserted into the optimized and validated tree available in ARB (Technical University of Munich, Munich, Germany; www.arb-home.de) using the maximum parsimony criterion and a special ARB parsimony tool that did not affect the initial tree topology to confirm BLAST affiliations. 16S

rRNA gene sequences were deposited in EMBL-GenBank under accession numbers AM849589 to AM849614.

Quantitative-PCR assays. Quantitative PCR was used to quantify six bacterial clades, i.e., *Chloroflexi* with one set of general GNSB941F-GNSB1340R [39, 40] and 2 sets of clade-specific primers; *Acidobacteria* with 2 sets of clade specific primers, and *Actinobacteria* with one set of primers. Primers from specific clades were designed from the bacterial sequences obtained in a previous study (see more details in [35]). DNA of ten random specimens for each of the eight populations (except for the population of Cap de Creus where only 8 specimens were found) was extracted from around 4 mm^3 of mesohyl using DNAeasy Blood and Tissue (Qiagen). We followed the manufacturer's instructions except for a 5 min incubation time before elution in a total volume of 75 µl supplied buffer. DNA concentrations were then determined by Qubit® Quantitation (Invitrogen). The assays were conducted on a Stratagene Mx3005P QPCR system. Each 25µl reaction contained 12.5 µl of 2x Brilliant SYBR® Green QPCR Master Mix (Stratagene), 0.4 µM of each primer, 125 mM of BSA (Promega), 30 nM of ROX reference dye, and 2 µl of template DNA at the optimal dilution. PCR conditions were 10 min at 95°C, followed by 40 cycles of 95°C for 1 min, 30 s at 64°C for the *Acidobacteria* runs and at 58°C for the other primers, and 72°C for 1 min, and finally all PCR products were subjected to a melting curve analysis to verify the specificity of the amplification. As various assays were run given the number of samples, we adjusted the fluorescence threshold to 0.099 to allow the comparison of all of the runs and the quantification with standard curves [35]. Adjusted threshold cycle (Ct) values were thus averaged over triplicates for each sample. Negative control included three reactions without DNA as control for contamination. Primer specificity was confirmed by running amplicons on agarose gel and by melting curve analyses. Copy numbers per µg DNA were calculated from the Ct value according to the standard curve previously obtained [35], the DNA

concentrations determined by Qubit® Quantification (Invitrogen) and the dilution applied. As the six targeted bacterial groups achieved most of the bacterial assemblage, we calculated the relative abundances as the ratio between the measured copy numbers for each group-specific qPCR assay to the total copy number obtained for the 6 assays in each specimen. For the life-stage comparison, only the 5 group-specific primers were used.

Statistical Analysis. We run statistical calculations available in the PRIMER v3.1 computer program [41] to analyze differences in bacterial community as a function of sponge population. Square root data were used to calculate Bray-Curtis similarity. Community dissimilarities were visualized by cluster analysis using a single linkage clustering model and non-metric multidimensional scaling (MDS) plots, and statistically tested by Analysis of Similarity (ANOSIM). An exploratory similarity breakdown using the SIMPER procedure was used to quantify the relative contribution of bacterial bands to dissimilarities, and the pairwise bacterial dissimilarities between pair of sponge populations.

We compared quantitative differences in bacterial-clades proportion between the sponge populations using MANOVA on rank-transformed clade relative abundances. We used *Post hoc* Tukey HSD to test for differences between populations. We also used a randomized block design multivariate analysis of variance (MANOVA) on rank transformed data, to test whether clade proportions differ as function of life stages and sponge specimen, both in Marseille (embryos vs. adults) and Els Bullents (larvae vs. adults).

RESULTS

As assembling data from different DGGE gels lead to significant gel effect (One way ANOSIM, global R=0.725, p=0.001, data not shown), DGGE gels were analyzed individually in all the pairwise combinations. The number of DGGE bands in the fingerprints was

relatively similar among samples (25-27 bands) and cluster analyses assigned sponge specimens from the same population mostly into the same cluster (see two examples in Fig. 3, where one specimen of Arenys clustered with Cap de Creus). Overall the core bacterial assemblage for the 8 sponge populations analyzed showed over 60% similarity, being the bacterial assemblage in Ceuta's population the most heterogeneous (SIMPER analysis, average similarity 71%), followed by Arenys, Cabrera, and Cap de Creus (78, 79, and 85%, respectively). From the set of DGGE gels, we selected 26 bands for 16S rRNA gene sequencing covering >85% of total band intensities. Sequences showed between 92 to 100% identity to nucleotide sequences previously deposited in Genbank, and were all affiliated with uncultured bacterial symbionts of invertebrates (mainly sponges). Overall, we found that *Chloroflexi* (13 sequences) and *Acidobacteria* (11 sequences) phyla were the most abundant groups in the DGGE gels. One sequence matched *Actinobacteria* (98% identity with sequences obtained from the sponges *Xestospongia muta* and *Ircinia felix)*, and the remaining sequence was a *Bacteroidetes*, again, closely related to an uncultured *Ircinia felix* endobiont (97% identity). Interestingly, several sequences were allocated in two distantly related clades previously reported within *Chloroflexi* (CL-1 and CL-2, respectively) and within *Acidobacteria* (AC-1 and AC-2, respectively).

Quantitative-PCR analysis of *Spongia*-associated bacteria. Using qPCR analysis, we observed significant variations in the relative abundance of the main bacterial clades among sites (MANOVA, Wilks' Lambda $F=8.623$, $p<0.001$; Fig. 4, and see absolute values in supplementary Fig. S2). All sponge specimens showed the same proportion of *Chloroflexi* using the general primers set ($F=1.525$; $p=0.173$; Fig. 4). However, we observed consistent differences at the clade specific level. Thus, the Atlantic population was significantly more enriched (relative abundance) in CL-1 than specimens from Arenys, Cabrera, and Ceuta (Fig.

4). Concerning the *Acidobacteria,* the clade AC-1 was more enriched in Arenys, Cap de Creus, and Pharillon populations, and less abundant in the Atlantic, whereas AC-2 was more homogeneously distributed. AC-2 was significantly more abundant in La Ciotat and less abundant in the Atlantic (Fig. 4). *Actinobacteria* had a significantly higher relative abundance in Portugal, Arenys, and Cap de Creus than in Cabrera (Fig.4).

Bacterial relative abundance in adults, embryos, and larvae. Significant quantitative differences were observed in the bacterial community amplified by qPCR both among life stages (adults vs. embryos, Wilks' Lambda $F = 14.741$, $p<0.001$, Fig. 5 and see more details in supplementary Table S1). Univariate tests revealed that only the *Actinobacteria* clade differed between the adults and their embryos ($F = 36.957$, $p < 0.001$). In Els Bullents, we found significant quantitative differences in the bacterial community amplified by qPCR among life stages (adults vs. larvae, Wilks' Lambda $F = 10.829$, $p = 0.019$), but not between sponge specimens. Univariate tests revealed that only the *Actinobacteria* clade differed between the adults and their larvae ($F = 11.270$, $p = 0.010$).

We also detected quantitative differences in the adult bacterial community between Marseille and Els Bullents (MANOVA, Wilks' Lambda $F = 4.663$, $p = 0.044$). *Acidobacteria* clade 2 and *Actinobacteria* clade were responsible for the differences (Univariate tests, *Acidobacteria* clade 2: $F = 9.441$, $p = 0.012$; *Actinobacteria*: $F = 13.06$, $p = 0.005$, Fig. 5).

DISCUSSION

Until quite recently, sponge associated microorganisms were believed to form stable communities across spatial and temporal scales [16, 18, 30]. However, more recent studies have challenged the uniformity and stability of sponge- symbiont associations. Lack of replication and/or absence of relative quantitative comparison may have contributed to

underestimate variability and thus diversity of bacteria within sponges. Most sequences available in bacterial genes libraries were obtained from 1 to at most 3 individuals of each species or locations. Nowadays, several studies show evidence that ecophysiological and environmental factors may also influence bacterial variability [17, 20, 23]. Although we found a common pool of bacteria inhabiting *S. lamella* (assemblages similarity, c.a. 60%), we also observed significant variations in the DGGE fingerprints. Although sponge specimens in a population clustered together according to their bacterial composition, we highlighted variations among the bacterial assemblages both within and among sponge populations.

Despite some well-known limitations inherent to the DGGE method and its detection limits [38, 42], this culture independent approach offers still a good compromise for the rapid detection and identification of predominant microbial species and simultaneous comparison among various samples. However, inter-gel comparison can be difficult [18, 19] especially dealing with highly diverse samples. In our study, gels were individually compared to avoid misinterpretation due to gel effects. Most studies dealing with bacterial diversity in sponges using DGGE have focused on presence/absence data. Qualitative estimations are appropriate for assemblage comparison from several host species [31, 43], but might underestimate variation when comparing specimens of the same host species. Signal intensity of DGGE bands can be applied to provide a rough overview of relative abundance changes from predominant bacterial groups [38]. We were cautious in the number of PCR cycles run to minimize the "plateau" phase and all samples were run under the same PCR and DGGE conditions. Thus, if there was any PCR bias it should be the same in all lanes and samples, and therefore comparison among samples is still valid. We were well aware that we worked with normalized relative abundances for comparison among samples, and not with total abundances. However, the recent application of Next Generation Sequencing approaches are

significantly expanding the knowledge in bacterial/invertebrates interactions (e.g. [44, 45]), and will overcome some of the known limitations of fingerprinting methods.

Sequences retrieved from the DGGE gels indicated that they were all related to uncultured bacteria and distantly related to the typical planktonic counterparts commonly found in previous studies reported in the literature [e.g., 46]. Bacteria inhabiting *S. lamella* resembled those in high-microbial-abundance sponges previously analyzed [11]. Most sequences belonged to *Chloroflexi* and *Acidobacteria.* These two phyla and *Actinobacteria* are the groups most frequently retrieved from 16S rRNA gene surveys in sponges [6] among the 15 bacterial phyla so far identified [11]. Within the *Chloroflexi* and *Acidobacteria*, we observed two distantly related clades within the same phylum specifically containing marine sponge sequences. *Acidobacteria* clade 1 gathered together bacterial assemblages from *Ircina felix* and *Smenospongia aurea* [7, 29]; whereas *Acidobacteria* clade 2 included sequences from *Aplysina fulva*, *Xestospongia testudinari*, and *Desmacidon* sp. [14, 46]. *Chloroflexi* clade 1 contained bacterial sequences from *S. lamella,* and from various sponge species such as *Ircina* and *Aplysina* [6, 7, 14, 16, 21]. The *Chloroflexi* clade 2 contained also bacterial sequences retrieved from diverse sponge species [11, 15, 29]. Interestingly, sequences excised at the same position in the gel and sequenced to confirm they were identical had also 100% identity to sequences obtained from *Aplysina aerophoba* collected in the same area in the NW Mediterranean [48], and 99% identity to a sequence from *Ancorina alata* collected in Northeastern New Zealand [49]. Further phylogenetic data with longer sequences may confirm whether it corresponds to the same symbiotic *Chloroflexi* species.

The sequence affiliated to Actinobacteria grouped within a sponge-specific cluster including *Actinobacteria* sequences from sponges collected in several oceans (*Ircina felix*, *Xetospongia muta*, *Xestospongia*, *testudinari*, *Aplysina aerophoba*, *Svenzea zeai*, and

Ancoriana alata). Sequence 6d11 (AM849590) was closely related to *Bacteroidetes* sequences stemmed from marine sponges (*Geodia barretti, Ircinia felix I. strobilina, Desmacidon* sp., and *Ancoriana alata,*), and sequences from this phylum have been recently retrieved from various sponge species, although clear phylogenetic affiliation remains still ambiguous. Longer 16S rRNA gene fragments and powerful treeing methods are needed to properly allocate this group.

In agreement with previous data [35, 50] we observed by qPCR analyses that *Chloroflexi* was the most abundant bacterial phylum within the sponge mesohyl. Relative abundance of *Chloroflexi* amplified by general primers seemed relatively stable in all sponge populations. However, relative abundance of *Chloroflexi* CL-1 and CL-2 varied significantly between sponge populations and mainly between Mediterranean and Atlantic populations. These two specific primers did not cover the large abundance of *Chloroflexi* obtained with the general primers, suggesting a larger diversity of *Chloroflexi* in *S. lamella* than in other sponge genera [35]. Certainly, additional Chloroflexi groups not detected by DGGE excised bands may inhabit the sponge mesohyl and deserve further investigations. Portugal population displayed significant differences, harboring significantly more CL-1 and less CL-2, whereas proportions of these 2 clades were similar in the remaining populations. Portugal also showed significantly less *Acidobacteria* and more *Actinobacteria* compared to other populations. Portugal population was the only sample obtained both from the Atlantic Sea and inside a marine cave. Unfortunately we cannot split the two effects with the current experimental design and any effect these features may have on the sponge bacterial composition remains to be determined. In fact, it is well-known that changes in the host physiology (such as transfer in aquarium and cultivation under artificial conditions, or disease outbreaks, [21-23] or abiotic factors such as environmental stress associated with metal pollution or light [20, 50] can

modify microbial assemblages. Larger bacterial diversity in stressed or sick sponges may be linked to a greater diversity of ecological niches available for bacteria and an increase of nutrients following sponge cells decay [23].

In this study, we also described the presence of embryos and larvae and compared the relative abundances of main bacterial groups between different life stages of the sponge using qPCR analyses. We provided information on the proportions of *Chloroflexi, Acidobacteria and Actinobacteria* found in *Spongia lamella* and we hypothesized they were mainly vertically transmitted to the next generation, as it has been reported in several sponge species [7, 15]. We observed both similarities and differences in bacterial community between adult sponges and their relative offsprings suggesting complex pathways for symbiont transmission as previously reported in *Hippiospongia* and *Spongia* species [26, 52]. In brooding sponges, nurse cells charged with endosymbiotic microbes engulfed from the mesohyl establish cytoplasmic bridges with the zygote membrane, transferring microorganisms and nutrients. When zygotes became embryos, they developed a cell follicle though also kept connected to the surrounding tissue by a system of radiating mesohyl bridges. These bridges probably facilitate the anchoring and/or feeding of the embryos during development and also allow symbiotic bacteria to migrate from the adult mesohyl to the intercellular spaces of the embryos [26]. Sponge adults and their respective embryos and larvae harbored the same relative proportions of *Chloroflexi* and *Acidobacteria* clades. However, the proportion of *Actinobacteria* was significantly higher in both embryos and larvae than in their respective parents. If a portion of the microbial community migrated from the adult mesohyl to embryos, higher proportion of *Acidobacteria* could simply be the result of a faster cell multiplication. Once the larvae settled, environmental conditions affecting both host fitness and microbial assemblage may balance relative abundances of the different populations [24].

Parenchymella larva, the most common larval type in demospongiae, is commonly classified among the lecitotrophic (non-feeding) meroplankton with limited swimming abilities [53]. They are self-sustained by using stored material such as lipid inclusions or bacteria phagocytosis [54]. Several studies highlight the importance of bacteria stored into larvae as food source [26, 52, 54]. In contrast in *Halisarca dujardini johnston* there is no evidence of lysis or digestion of bacteria at any stage of the development, although symbiotic bacteria were present intercellularly in cleaving embryos and larvae [27]. Furthermore, brooded larvae that develop using yolk (lecitotrophs) are typically larger, easier to see and lack morphological characteristics that decrease fish predation compared to longer-lived planktotrophic larvae. Brooded larvae of many diverse sessile invertebrates are commonly distasteful to benthic fishes, and their unpalatability has often been attributed to chemical defenses [55]. Among bacteria, *Actinobacteria* are known as one of the richest sources of active secondary metabolites [56-60]. Thus, the presence of *Actinobacteria* in the embryos and larvae might serve either as food source for the *S. lamella* lecitotrophic larva or as chemical defense against potential predators, competitors, foulers, or infectious microorganisms.

Overall, although the final causes for the variability found in this study remain unclear, some assumptions can be set out, and a combination of horizontal (environmental) and vertical (parental) transmission may be considered [11, 61]. A fraction of bacteria environmentally transmitted may result from “selective enrichment” of specific bacteria that remained undetectable in the surrounding plankton [16], and sponges must provide a favorable habitat for them to grow over the methodological detection thresholds. Differences between life stages may also be the result of transient bacteria acquired from the surrounding water, or by a differential and preferential bacterial transmission. Competition and selection,

which are dynamic processes, affect the structure of the microbiota [62] and could also explain variability in bacteria associated to sponge. Genetic variability of sponge populations could also contribute to the observed variability in bacterial communities [24]. As most bacterial groups appeared consistently through *S. lamella* populations and main bacterial groups were present in sponge populations, a combination of "filtration-concentration" (which depend on sponge physiology and environmental factors and are more proned to variations) and "hereditary-colonization" (linked to true symbionts at the origin of the uniformity among sponge associated bacteria) may be possibly accounting. These results might correspond with multiple levels of specificity through which bacteria are associated to sponges, and should be carefully considered for a more accurate understanding of sponge microbial ecology.

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Figure Legends

FIG. 1: Locations of *Spongia lamella* sampling sites in NW Mediterranean Sea and Atlantic Ocean. A zoom on the area of Marseille is shown.

FIG. 2: *Spongia lamella* life stages including (a) brooded embryos gathered within the parent sponge (bar: 0.5 cm), as: aquiferous system; bc: brooding chamber, emb: embryos from distinct brooding chambers into the adult mesohyl; and (b) newly released parenchymella larva observed under light microscopy (bar: 500µm).

FIG. 3: Cluster diagrams of DGGE fingerprints of bacterial assemblages in *Spongia lamella* at (a) local and (b) regional scales. Six sponge specimens were analyzed per site.

FIG. 4: Relative abundance as estimated by qPCR analysis of *Chloroflexi* (GNSB-941F, Cl1, and Cl2), *Acidobacteria* (Ac1 and Ac2), and *Actinobacteria* (Actino) in several populations of *Spongia lamella*. Vertical bars are standard errors (n=10 samples per population except Cap de Creus n=8). ANOVA on rank-transformed proportion of clade relative abundance, p-values and *Post hoc* Tukey HSD included.

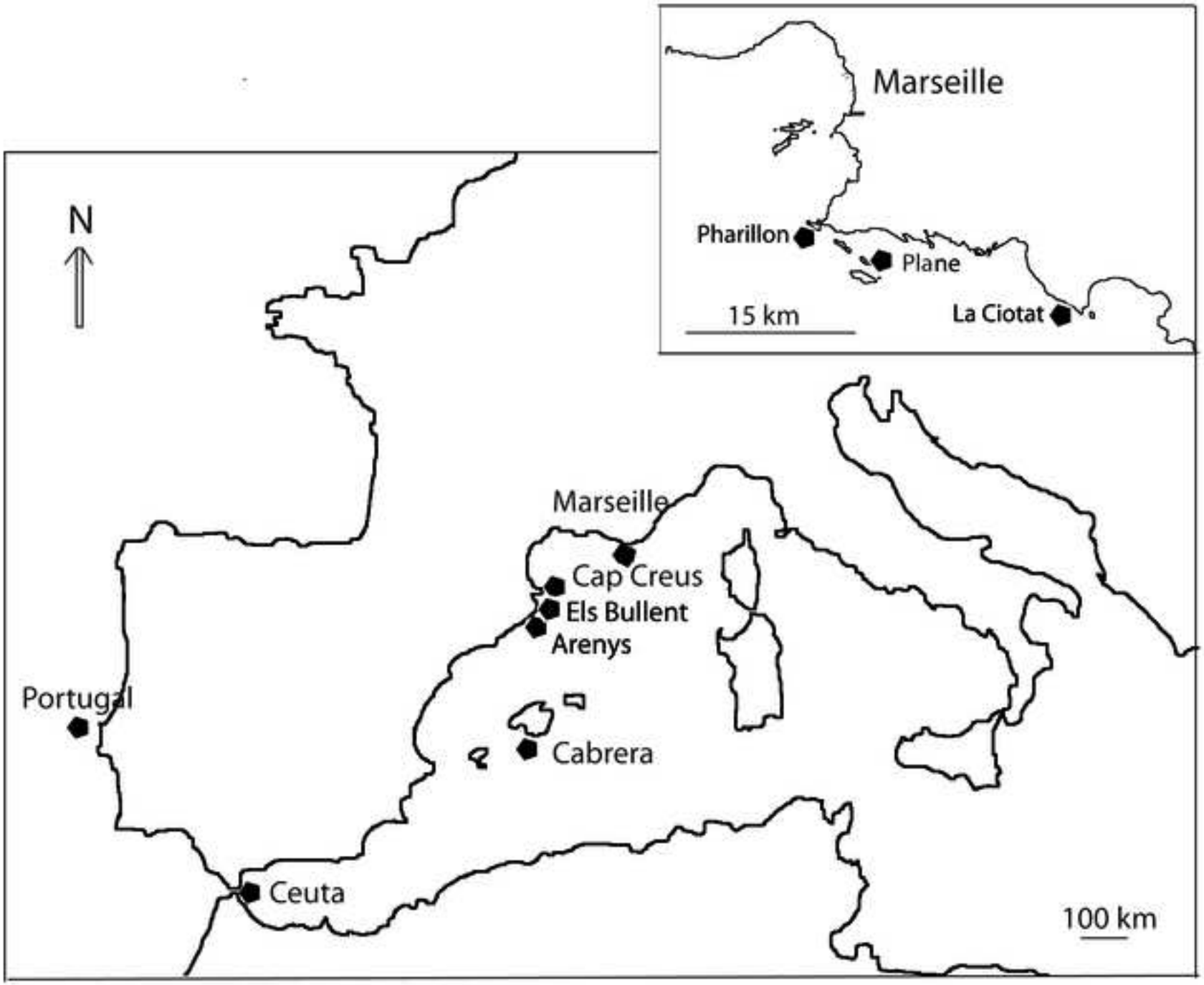
FIG. 5: Relative proportion as estimated by qPCR analysis of *Chloroflexi* (clades Cl1, and Cl2), *Acidobacteria* (clades Ac1 and Ac2), and *Actinobacteria* (clade Actino); in *Spongia lamella*, from adults (A_emb) and embryos (emb) sampled in Marseille; and adults (A_larv) and larvae (larv) sampled in Els Bullents. Vertical bars are standard errors. P-value from the randomized block (Multivariate) Analysis of Variance included. NS: non significant.

Supplementary figures

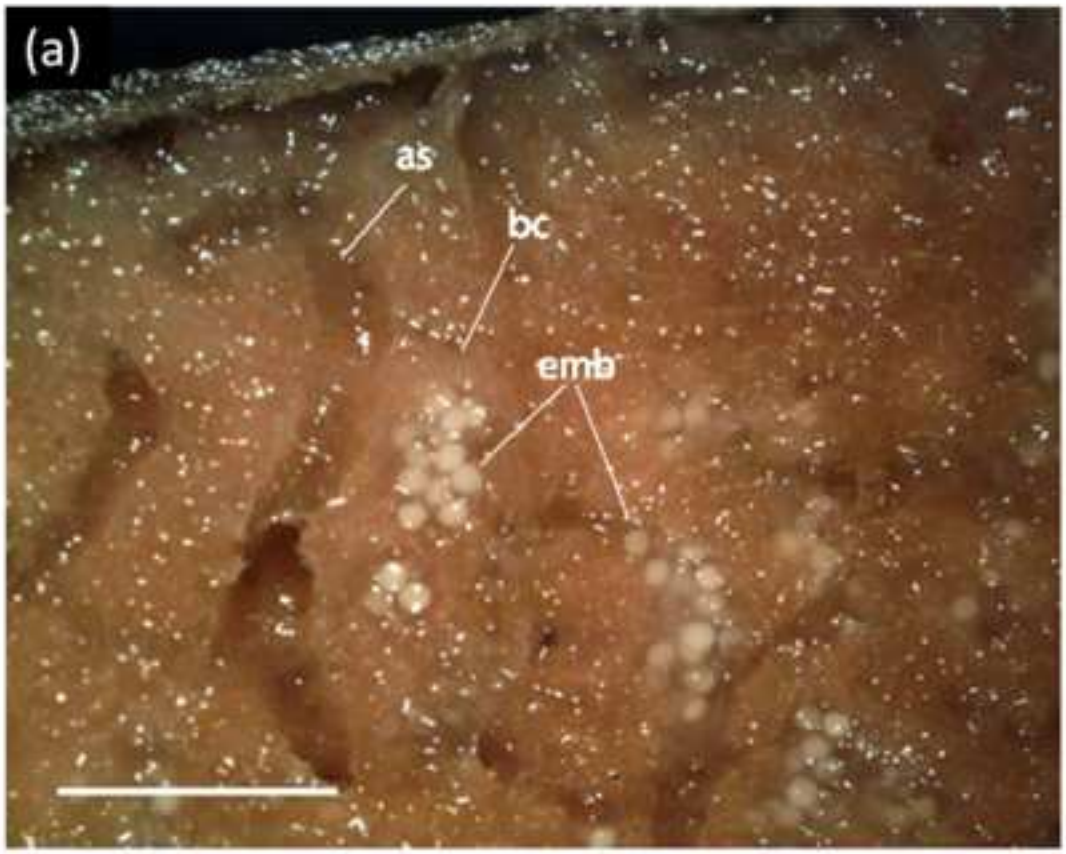
Fig. S1. Negative images of DGGE gels obtained in this study

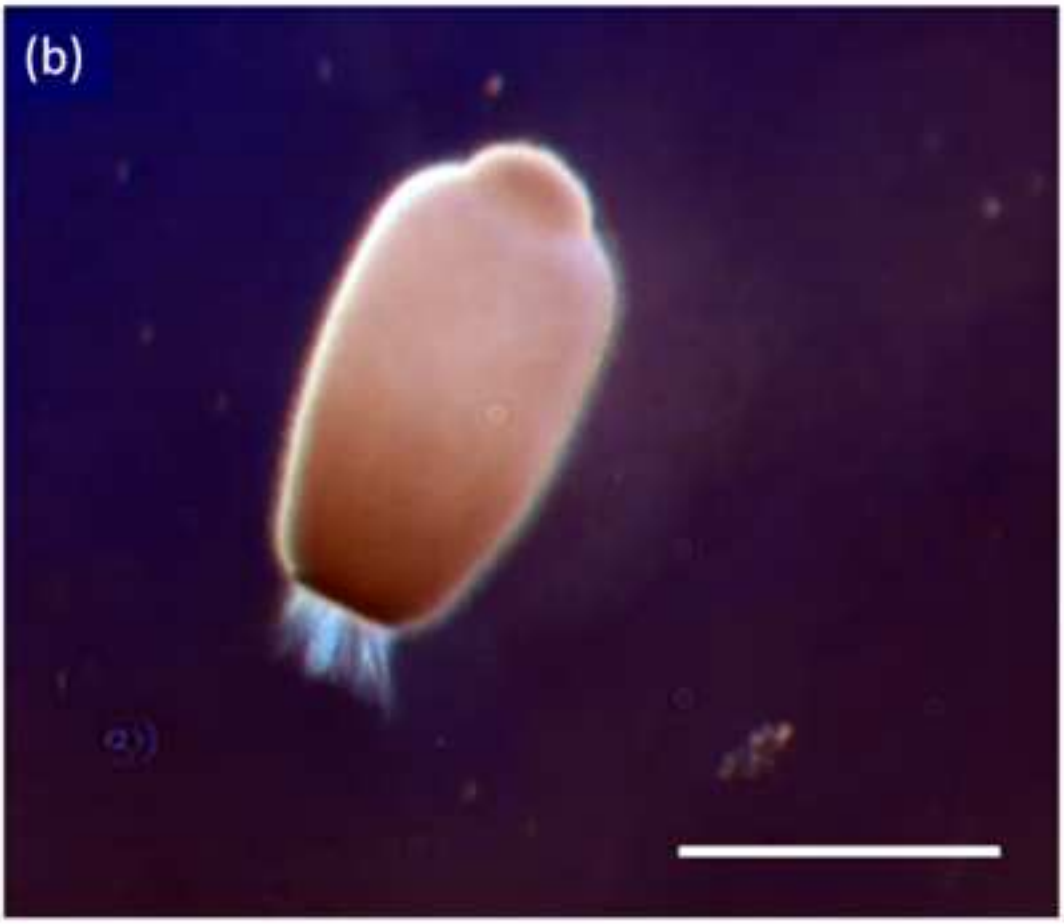
Fig. S2. Abundance as estimated by qPCR analysis of six bacterial clades in several populations of *Spongia lamella*, i.e., *Chloroflexi* (GNSB-941F, Cl1, and Cl2), *Acidobacteria* (Ac1 and Ac2), and *Actinobacteria* (Actino).

Figure

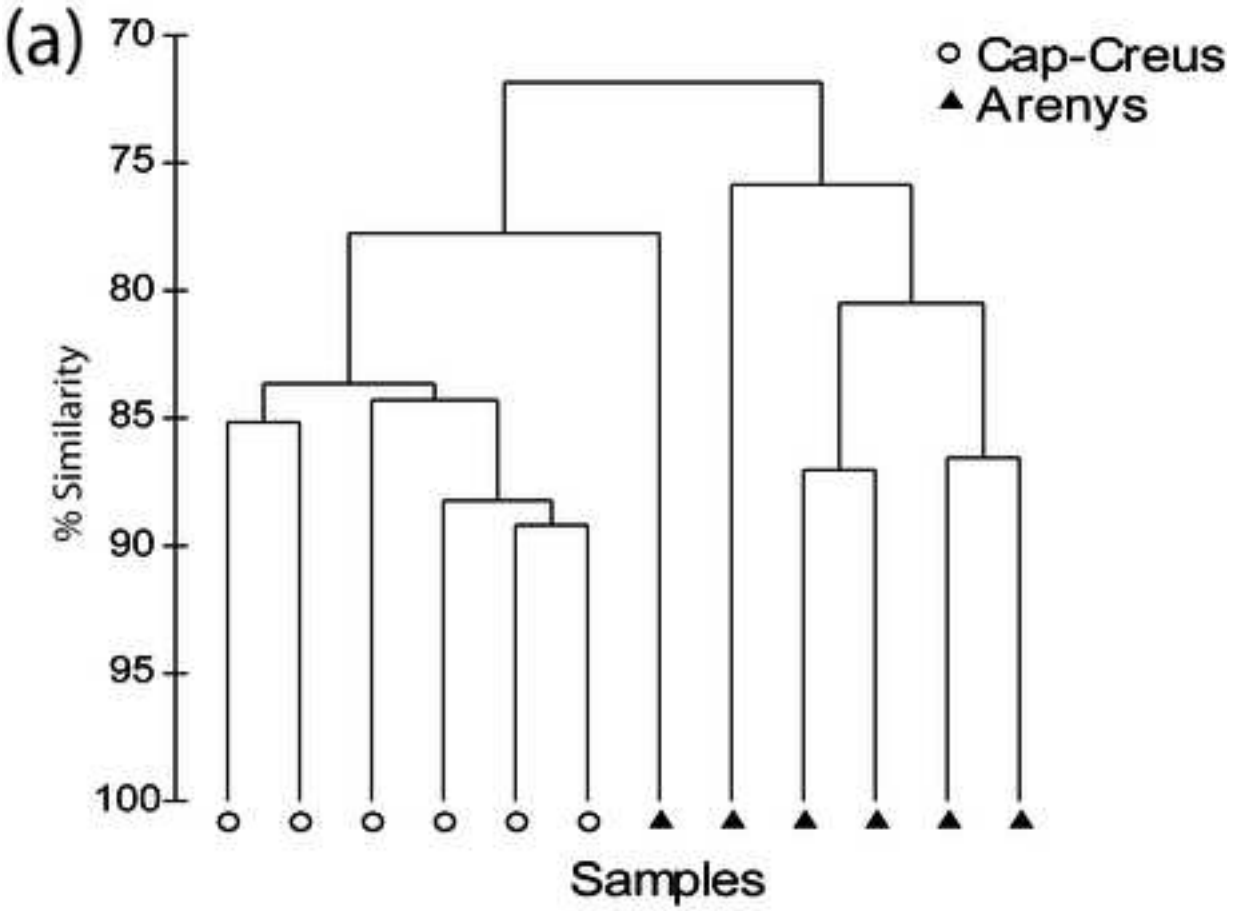

Marseille
Pharillon
Plane
15 km
La Ciotat
N
Marseille
Cap Creus
Els Bullent
Arenys
Portugal
Cabrera
Ceuta
100 km

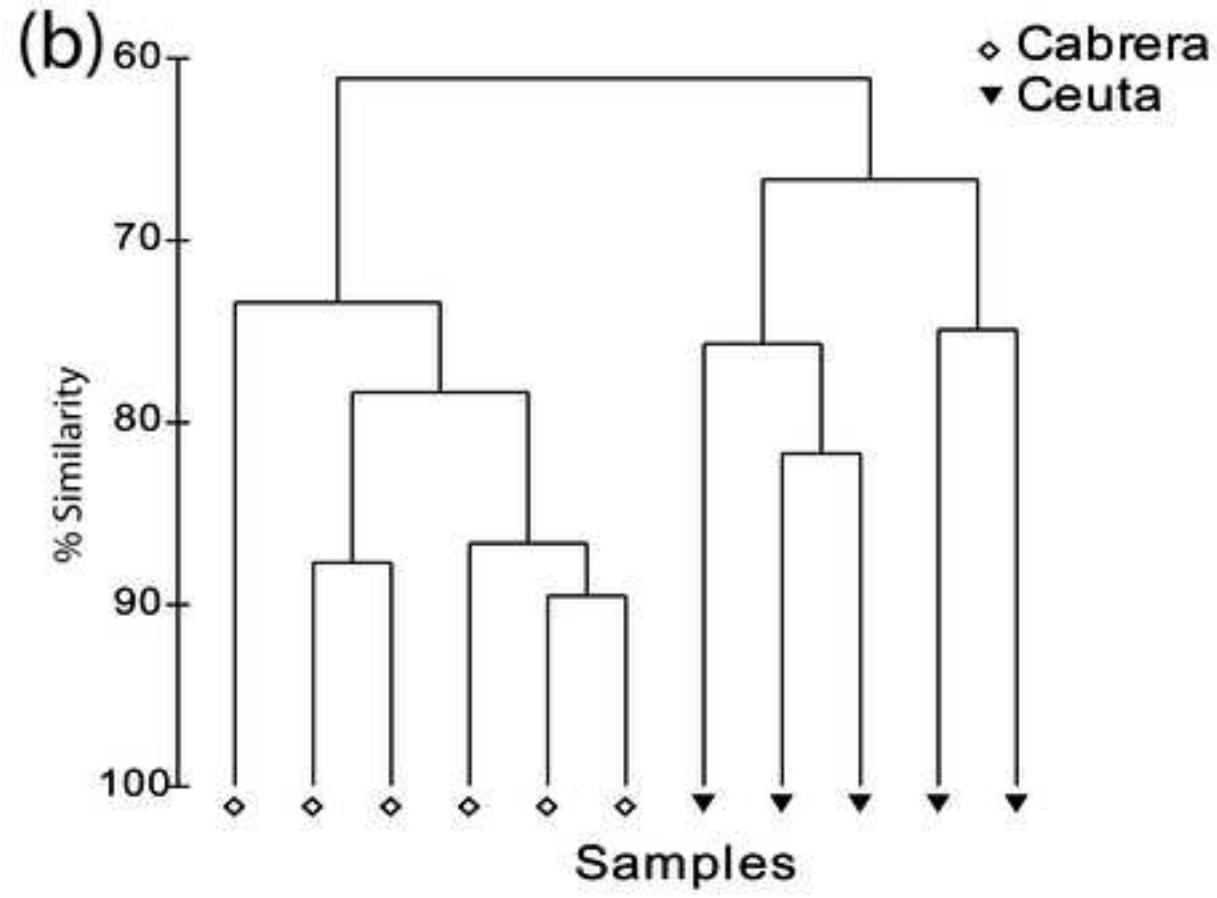
Figure



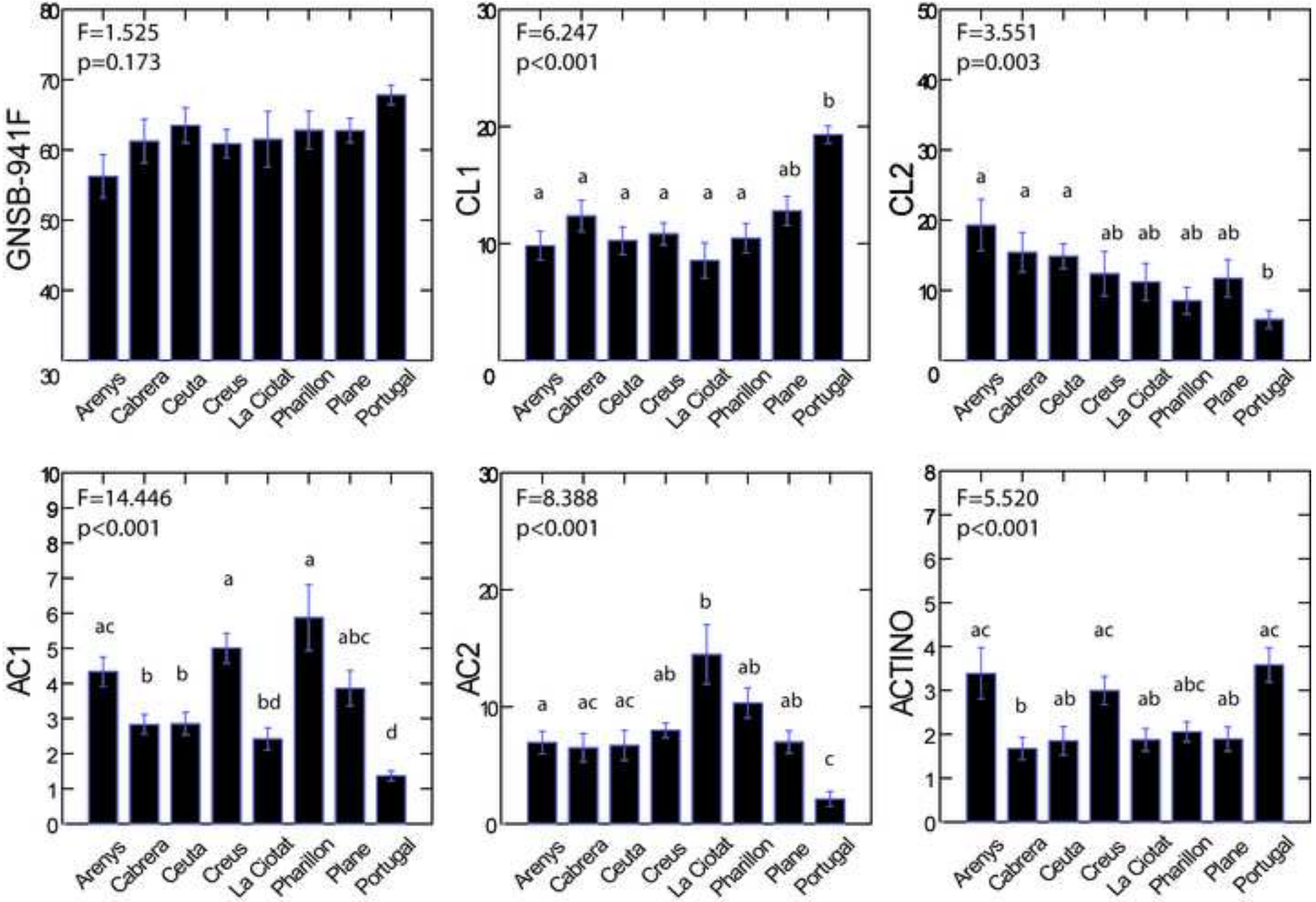


Figure


(a)
70
75
80
85
90
95
100
% Similarity
Cap-Creus
Arenys
Samples


(b)
60
70
80
90
100
% Similarity
Cabrera
Ceuta
Samples

Figure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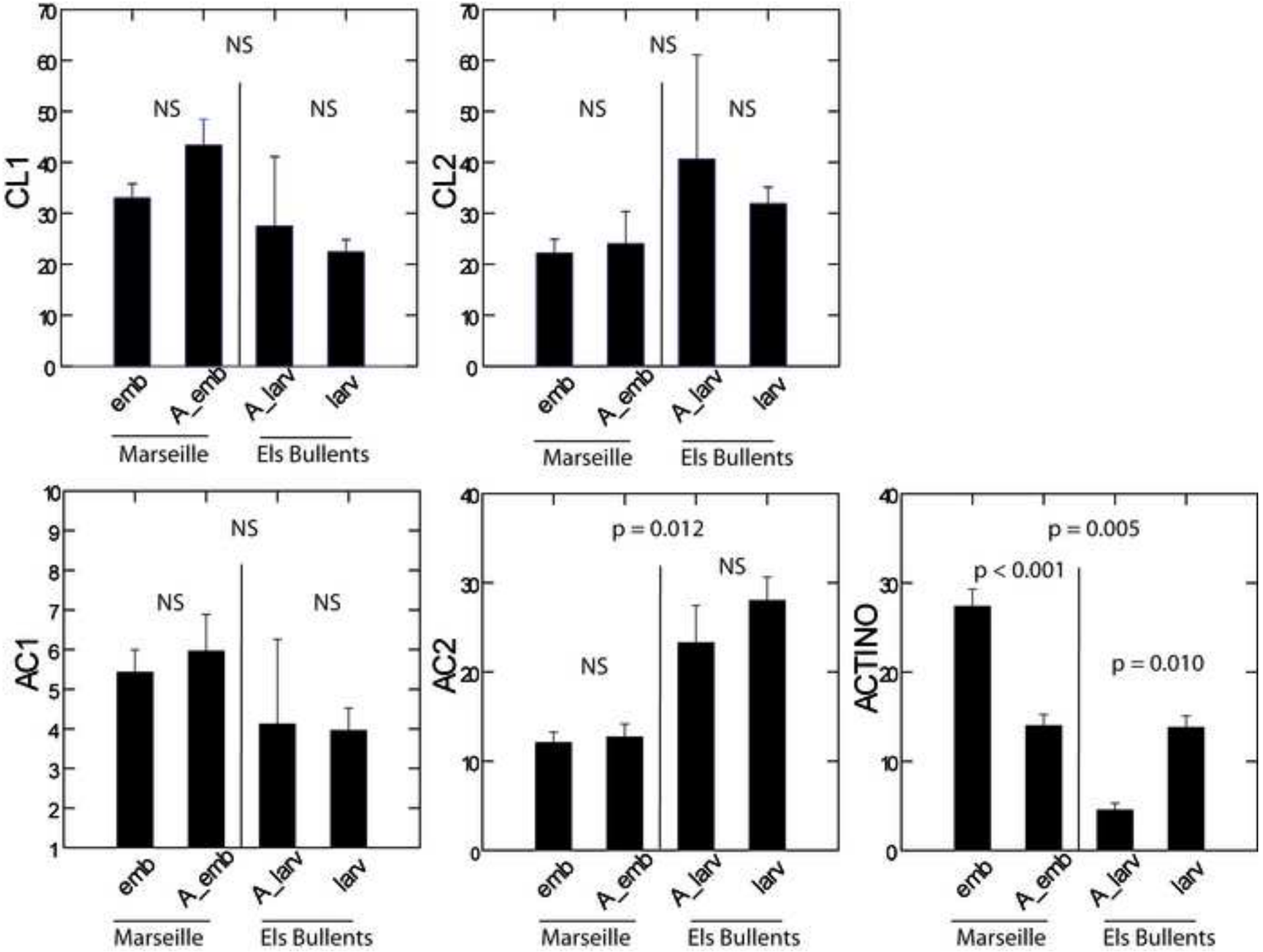
F=1.525
p=0.173
CL1
F=6.247
p<0.001
CL2
F=3.551
p=0.003
AC1
F=14.446
p<0.001
AC2
F=8.388
p<0.001
ACTINO
F=5.520
p<0.001
Arenys
Cabrera
Ceuta
Creus
La Ciotat
Pharilion
Plane
Portugal

Figure


CL1
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emb
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larv
Marseille
Els Bullents
CL2
NS
NS
NS
emb
A_emb
A_larv
larv
Marseille
Els Bullents
AC1
NS
NS
NS
emb
A_emb
A_larv
larv
Marseille
Els Bullents
AC2
p = 0.012
NS
NS
emb
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A_larv
larv
Marseille
Els Bullents
ACTINO
p = 0.005
p < 0.001
p = 0.010
emb
A_emb
A_larv
larv
Marseille
Els Bullents