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High diversity of skin-associated bacterial communities of marine fishes is promoted by their high variability among body parts, individuals and species

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Abstract :

Animal-associated microbiotas form complex communities, which are suspected to play crucial functions for their host fitness. However, the biodiversity of these communities, including their differences between host species and individuals, has been scarcely studied, especially in case of skin-associated communities. In addition, the intraindividual variability (i.e. between body parts) has never been assessed to date. The objective of this study was to characterize skin bacterial communities of two teleostean fish species, namely the European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*), using a high-throughput DNA sequencing method. In order to focus on intrinsic factors of host-associated bacterial community variability, individuals of the two species were raised in controlled conditions. Bacterial diversity was assessed using a set of four complementary indices, describing the taxonomic and phylogenetic facets of biodiversity and their respective composition (based on presence/absence data) and structure (based on species relative abundances) components. Variability of bacterial diversity was quantified at the interspecific, interindividual and intraindividual scales. We demonstrated that fish surfaces host highly diverse bacterial communities, whose composition was very different from that of surrounding bacterioplankton. This high total biodiversity of skin-associated communities was supported by the important variability, between host species, individuals and the different body parts (dorsal, anal, pectoral and caudal fins).

Keywords : *Sparus aurata*, *Dicentrarchus labrax*, skin microbiome, next generation sequencing, phylogenetic diversity

1. Introduction

All animals host at their surface and in several internal organs consortia of microorganisms, namely bacteria, archaea, fungi and viruses, collectively called microbiotas. These microbiotas form diversified communities and play critical roles for their host, as they facilitate nutrient absorption, regulate metabolism, and defend against pathogen invasion (Sekirov *et al.*, 2010).

Skin habitat is a unique interface, influenced both by surrounding environment (air, water, soil) and host-associated factors (health state, mobility, excretion of wastes and mucus, and immune molecules secretion). These interacting factors lead to a patchy physical and chemical environment at the surface of an individual and to contrasted environments between individuals (Shephard, 1994; Grice and Segre, 2011). Therefore a variability of skin microbiome in terms of abundance and diversity is expected at both inter- and intra-individual scales. Human skin microbiota has been particularly well studied, especially since the launch of the Human Microbiome Project in 2007 (Turnbaugh *et al*., 2007). These studies highlighted the high diversity of human skin microbiota (Schommer and Gallo, 2013). They also evidenced that human skin-associated bacterial communities were highly variable between body parts, and between individuals (Fierer *et al.*, 2010). These inter- and intra-individual variations have been related to individual physiology (e.g. age, sex, health state, immune system), personal habits (e.g. hygiene, cosmetic use, clothing), and local-scale parameters (e.g. pH, temperature, humidity), even if the specific impact of each of these drivers, and the underlying interactions at a microbial scale were not systematically demonstrated (see Grice and Segre, 2011, for a comprehensive review).

In contrast to human, skin microbiotas of animals are yet still largely unknown. Among them, marine vertebrates, which represent more than 10 000 species on Earth (www.iobis.org), were only occasionally investigated during the last two decades (Larsen *et al.*, 2013). In addition, most of the recent studies on marine vertebrates focused on the gastrointestinal microbiome (Mouchet *et al.*, 2012; Xing *et al.*, 2013), and revealed tight interactions between the host and its gut microbial communities (Pérez *et al.*, 2010). Bacterial epibionts of marine vertebrates remains largely understudied, yet they are believed to play major roles in maintaining host health (Boutin *et al.*, 2012). The few reports published to date found that the bacterial community composition was different among six Atlantic teleostean fish species, and highly different from that of surrounding planktonic communities (Larsen *et al.*, 2013). Similarly, a recent study, focusing on wild humpback whale skin-associated bacterial communities, evidenced that despite individuals share a core set of species, bacterial community composition was variable between individuals because of differences in host physiology (Apprill *et al.*, 2014). Moreover, while the entire fish body, including the head, trunk, and also the fins, is recovered by the same integument, body parts of marine fishes may harbor contrasted local conditions due to *(i)* disparate epidermal mucous composition throughout body's surface (Ángeles Esteban, 2012), *(ii)* variable exposure to nutrient excretion fluxes through gills and vent, and *(iii)* variable water flow during swimming. These environmental variations at fish surface may drive variations of skin-associated bacterial communities between body parts. Such differences in skin microbial diversity between body parts have never been assessed to date on marine animals. Additionally, the only studies that assessed marine animals skin microbial diversity focused on wild individuals or fish kept in *in situ* cages, making difficult to disentangle the effects of past and current environmental conditions experienced by the animals from their intrinsic characteristics (e.g. physiology, behavior) at the sampling time. Skin surface, and hence bacterial epibionts, are indeed directly exposed to the external biotic and abiotic components from the surrounding water column, while marine vertebrates are vertically and horizontally very mobile, which induces spatio-temporal variability (*i.e.* effects of seasonality and geographical location) in the composition of skin microbiota (Le Nguyen *et al.*, 2008; Wilson *et al.*, 2008). To quantify the variability of this

parameter among individuals and species independently from environmental variability, it is therefore necessary to use animals raised in the same environment.

Another current gap in the description of biodiversity of skin microbial communities is the lack of simultaneous assessment of both taxonomic (*i.e.* based on species or OTUs) and phylogenetic (*i.e.* based on phylogenetic lineages) diversity facets (Escalas *et al.*, 2013). Indeed, phylogenetic diversity has been proposed to be a better predictor of community functioning than taxonomic diversity because it accounts for complementarities among species (Zavarzin *et al.*, 1991; Fierer *et al.*, 2007). For instance, using marine bacterial species, Gravel and co-workers (2012) experimentally showed that the phylogenetic diversity of planktonic bacterial communities strongly explained the productivity of the community, suggesting functional complementarity of different phylogenetic lineages (even if functional conservatism along phylogenetic lineages is a debated issue ; see Achenbach and Coates, 2000, and Wellington *et al.*, 2003). Moreover, communities composed of distantly related bacterial species stabilize community production when they are exposed to perturbations (Awasthi *et al.*, 2014). Changes of the phylogenetic diversity of skin microbiome may therefore change its functions, and may thus disturb its homeostatic relations with the host and finally may favor disease. The phylogenetic diversity should then be considered when assessing the level and variability of skin microbiome diversity. For example, two communities dominated by different OTUs, *i.e.* having a high taxonomic structural dissimilarity will have a low phylogenetic dissimilarity if abundant OTUs are phylogenetically close.

In addition, each diversity facet (taxonomic and phylogenetic) should be assessed accounting not only for composition (species presence/absence) but also for the structure of community by considering species relative abundances. Indeed, two communities can appear to be highly dissimilar in terms of phylogenetic composition (*i.e.* they host phylogenetically very distant species) only because of their rare species, and thus be similar in terms of phylogenetic structure (*i.e.* when taking account of species relative abundances) (Escalas *et al.*, 2013).

In this study we assessed the interspecific, intraspecific and intra-individual variability of the taxonomic and phylogenetic diversity of skin bacterial communities of two marine fish species, namely the European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*), bred in controlled environmental conditions. Our first objective was to determine whether bacterial diversity differed between the skin-associated bacterial communities and the surrounding bacterioplankton. Our second aim was to test whether two fish species host different skin bacterial communities. Finally, we assessed the variability of bacterial diversity between individuals per fish species, and, within individuals, between different parts of the body (*i.e.* anal, caudal, dorsal and pectoral fins), and compared it with the interspecific difference. As mentioned above, an effect of host species has already been evidenced in other wild teleostean species. We expected that this important variability should persist in controlled conditions between seabass and seabreams-associated bacterial communities, because of intrinsic physiological differences between these species. Additionally, we expected a high level of intra-individual variability of fish skin bacterial associates, due to differences in habitats between the different body parts studied (Ángeles Esteban, 2012).

2. Materials & Methods

2.1. Sampling

Four European seabass (*Dicentrarchus labrax*) and four gilthead seabreams (*Sparus aurata*) were sampled at the Marine Station of University of Montpellier (Sète, France). After larval stage, the two species were raised in the same conditions in two mono-specific tanks (5 m^3) for 2.5 and 7 years, respectively. The two tanks were connected to the same water filtration system (activated carbon filter, no sterilization) in a closed circulating water system, and tanks were regularly filled with subsurface water of the Thau lagoon (renewal of 2% vol. per day). Physico-chemical conditions were almost identical in the two tanks at the time of sampling (see supplementary data S1). Individuals of the two species were fed with the same commercial pellets and received no anti- or probiotic treatment during their entire life. Individuals were hooked, suspended in air by the hook shaft, stunned and killed by cervical dislocation by a certified animal manipulator (following the European directive 2010/63/UE on the protection of animals used for scientific purposes). This protocol was chosen to avoid contacts between fish surface and other surfaces (tank wall, soil or hands of experimenters). Immediately after death, dorsal, caudal, left pectoral and anal fins were collected with ethanol-rinsed scissors and surgical pliers and placed into sterile cryotubes. Sex was determined by direct observation of gonads. There were three male seabass and one female, and three female seabream and one male. Two samples of 100 mL of tank water were collected in each tank and filtered through a 47 mm 0.2 µm polycarbonate membrane (Whatman, Clifton, USA). The four filters were then placed in sterile cryotubes. All samples were snap frozen at -196°C in liquid nitrogen, transported to the lab and stored at -80°C for one week before being analyzed.

2.2. DNA extraction, amplification and sequencing

Bacterial DNA recovery from fin surface was adapted from Amalfitano & Fazi (2008) for complex matrices. Each fin was immerged into 6 mL of a PBS solution containing 0.5% of tween 20 (vol/vol) and vortexed at maximum speed during 10 minutes (Vortex genie 2, Scientific Industries, Bohemia, USA). The solution was then filtered through a 47 mm 0.2 µm polycarbonate membrane (Whatman, Clifton, USA). Bacterial DNA was extracted by using the DNeasy® Blood & Tissue kit (Qiagen, Venlo, Netherlands), following the modified manufacturer"s protocol facilitating lysis of Gram-positive bacteria. DNA was eluted in 100 µL of buffer AE and quantified by fluorescence using the Qubit dsDNA BR Assay kit (Invitrogen, Carlsbad, USA) and the Qubit® 3.0 Fluorometer. Concentrations averaged 78.8 ng μL^{-1} (±9.6, *n*=36). DNA quality was assessed by spectrophotometry (Nanodrop 1000, Wilmington, USA). Values of A_{260nm}/A_{280nm} and A_{260nm}/A_{230nm} averaged 2.3 (±0.2) and 4.6 (±0.5), respectively. All DNA samples were then diluted to 10 ng μL^{-1}. An external laboratory (Research and Testing Laboratory, Lubbock, USA) performed PCR Amplification of the V1-V3 region of the 16S rRNA gene using universal bacterial primers 27F (5"-AGRGTTTGATCMTGGCTCAG-3") and 519R (5"-GTNTTACNGCGGCKGCTG-3") (Vergin *et al.*, 1998; Ishak *et al.*, 2011) and the HotStarTaq Plus Master Mix (Qiagen, Venlo, Netherlands) as follows: initial denaturation at 94°C for 3 min, followed by 28 cycles of 94°C for 30s, 53°C for 40s and 72°C for 1 min, ending with a final extension at 72°C for 5 min. After amplification, equimolar amounts of DNA were mixed, purified (Ampure beads kit, Agencourt Bioscience Corporation, USA) and sequenced using a Roche 454 FLX titanium pyrosequencer. We obtained a total of 104 548 (>200 bp) reads from the sequencing of PCR amplicons from the 36 samples. The nucleotide sequence data reported are available in the NCBI SRA database under the accession number SRP050454.

2.3. Sequence processing and taxonomic classification

Sequences were processed following the SOP analysis pipeline of (Schloss *et al.*, 2011; http://www.mothur.org/wiki/454_SOP, 02/2014) using Mothur (Schloss *et al.*, 2009). Briefly, anormal flows (homopolymers >8bp, >1 mismatch to the barcode, or >2 mismatches to the primer), and very short flows (<200 bp) were discarded. Then, sequences were determined using a maximum likelihood approach using PyroNoise (Quince *et al.*, 2011). Chimera were detected and eliminated using UCHIME (Edgar *et al.*, 2011).

Up to 71 744 unique sequences with an average length of 244 bp were retained. Sequences presenting more than 97% identity were clustered, and a representative sequence (*i.e.* the closest sequence of all other sequences) for each cluster was selected. Using these sequences, clusters were classified using the Ribosomal Database Project II Classifier (Wang *et al.*, 2007). Non-prokaryotes and mitochondrial clusters were excluded. The number of sequences varied between samples (see supplementary information S2) and these differences may not reflect true difference in richness and biomass of bacterial communities but rather difference in sampling effort (e.g. mucus volume) and/or efficiency of amplification and sequencing. To correct for this uneven number of sequences we calculated taxonomic and phylogenetic diversities on bootstrapped samples (Bryant *et al.*, 2008). More precisely, we considered 1 000 randomized subsamples of 113 sequences (the minimal number of sequences among the 36 samples) for each community. We then only considered the mean of diversity indices among the 1 000 bootstrapped samples because their variances were negligible.

2.4. Phylogenetic analyzes

All representative sequences were aligned using MAFFT v7 (FFT-NS2) (Katoh *et al.*, 2002) and a phylogenetic tree was reconstructed using FastTree 2 (Price *et al.*, 2010), implemented in QIIME software (Caporaso *et al.*, 2010). The tree was rooted using a set of eight archaeal 16S sRNA gene sequences obtained from SILVA database (Quast *et al.*, 2013). A chronogram was then adjusted on the phylogenetic tree using the „chronos" function (*discrete* model, 20 evolution rates) provided in the R-package *ape* (Paradis *et al.*, 2004). This function provides a dated ultrametric tree using a maximum likelihood algorithm and calibration points, provided in supplementary information S3.

2.5. Alpha diversity computation

Alpha diversity was described using a set of four complementary indices, describing taxonomic and phylogenetic compositional diversity (*i.e.* taxonomic and phylogenetic richness based respectively on presence/absence of OTUs and phylogenetic lineages), and taxonomic and phylogenetic structural diversity (*i.e.* taking account of relative abundances of OTUs and phylogenetic lineages, respectively).

Taxonomic richness (S) was assessed as the number of different OTUs in each community. Phylogenetic richness (Faith"s PD), based on the sum of branch lengths of the phylogenetic tree grouping OTUs present in the sample, was calculated using the *Picante* R-package (Kembel *et al.*, 2010). Taxonomic structural diversity was assessed using Shannon alpha diversity (Shannon, 1948). Phylogenetic structural diversity was assessed using Allen alpha diversity (Allen *et al.*, 2009). Allen index of diversity is similar to the Shannon diversity, excepted that it is based on phylogenetic branch lengths instead of OTUs. Allen index was calculated using the „ChaoPD" function of *entropart* package (Marcon and Hérault, 2014). These two indices were expressed in equivalent number of species, as recommended by

Jost (2007). This transformation allows direct comparisons between diversity values (Chao *et al.*, 2014).

2.6. Beta diversity computation

Alpha diversity indices describe diversity at a local scale. To fully assess bacterial diversity, it is also necessary to measure beta diversity, *i.e.* the dissimilarity between communities. Similarly to alpha diversity computation, dissimilarity was assessed using a set of four indices describing each facet (phylogenetic and taxonomic) and component (compositional or structural) of diversity.

Compositional (*i.e.* based on presence/absence matrices) taxonomic and phylogenetic beta diversities were assessed by the Sorensen (Sørensen, 1948; Koleff *et al.*, 2003) and phyloSor dissimilarity indices (Bryant *et al.*, 2008; Leprieur *et al.*, 2012), respectively, using the *betapart* R-package (Baselga and Orme, 2012). PhyloSor is similar to the Sorensen index, excepted that it is calculated on branch lengths. These two beta diversity measurements are scaled between 0 (when communities share the same OTUs or phylogenetic lineages) and 1 (when communities have no OTU or phylogenetic lineages in common).

Structural (*i.e.* accounting for entities relative abundances) taxonomic and phylogenetic beta diversities were calculated using the multiplicative decomposition of Shannon and Allen indices, respectively, following the general framework proposed by Chao and coworkers (2014). These two beta diversity measures were scaled between 0 (when, in case of taxonomic beta diversity, communities share the same OTUs at the same abundances) and 1 (when communities have no OTU in common) as suggested by Villéger and coworkers (2012), and were therefore directly comparable to Sorensen and PhyloSor indices (Chao *et al.*, 2014). These four beta diversity indices were calculated at the intra- and inter-individual, and inter-species scale.

2.7. Statistic analyses

Phylogenetic and taxonomic richness tend to be correlated as the increasing the number of OTUs increase the probability of covering more phylogenetic lineages. Consequently, we computed the Standardized Effect Size of the PD index *(SES.PD)* comparing the observed PD value and its expected value under a null model maintaining sample species richness, using the *SES.PD* function of the *Picante* package (Kembel, 2009). A positive/negative *SES.PD* value indicates a phylogenetic over-/under-dispersion, *i.e*. OTUs found in the sample are more/less phylogenetically distant than expected.

Effects of species, individual, sex, and type of fin on alpha diversity values were tested using Kruskal-Wallis tests, and subsequent pairwise comparisons were performed using post-hoc Man-Whitney tests (*pgirmess* package, Giraudoux, 2011).

Three independent non-parametric analyses (PERMANOVA) of the effect of host species, individuals, and body parts on the variability of the structure and composition of the microbiota (*i.e.* on each four beta diversity matrices) were performed using the „adonis" function of the *Vegan* package (Dixon, 2003).

To compare the community composition in each type of samples, Venn diagrams were constructed using eulerAPE (Micallef and Rodgers, 2014) and the R package *VennDiagram* (Chen and Boutros, 2011).

3. Results

3.1. Alpha diversity

Alpha diversity in water and on fish skin. Alpha diversity patterns differed, depending on the facet (taxonomic or phylogenetic) and component (composition or structure) considered. Indeed, the taxonomic richness (related to species composition) was significantly higher in each water replicate (averaging ca. 46 OTUs ± 2.3 after bootstrap subsampling, *n*=4 water replicates) than on fish skin (ca. 22 OTUs ± 7.2 per bootstrapped sample, *n*=32 ; but note there were 73 ± 5.4 OTU per individual) (Kruskal-Wallis test (KW), *P*<0.05, Fig. 1, a). On the contrary, taxonomic alpha diversity (accounting for relative abundances of OTUs) was not significantly different between the two types of samples (Shannon alpha diversity, KW *P*>0.05, Fig. 1 c). The high taxonomic richness of planktonic communities was indeed mainly due to rare OTUs (OTUs accounting for <1% of total abundance), representing about 89.9±0.8% of the OTUs present in water. On the contrary, OTUs abundances were more evenly distributed in skin communities, with fewer rare OTUs (4.0 ± 7.3% of present OTUs). Among all identified OTUs, only 7% were found in water replicates, while all of them were found in at least one fish sample.

Additionally, while phylogenetic richness (PD) did not significantly differ between water and fish (KW, *P*>0.05, Fig. 1 b), phylogenetic alpha diversity (Allen alpha diversity, based on the relative abundances of phylogenetic lineages) was significantly lower in water than in fish skin samples (KW, *P*<0.05, Fig. 1 d). Besides, planktonic communities were significantly phylogenetically under-dispersed, *i.e.* the OTUs forming the community were clustered on the phylogenetic tree (*SES.PD* = -2.95 ± 1.8, *P* = 0.02 ± 0.01), while fish communities were neither under- nor over-dispersed (*SES.PD* = -0.36 ± 0.8, *P* = 0.39 ± 0.2).

Alpha diversity patterns in fish skin samples. For all facets and components of the alpha diversity, there was no significant difference between *i)* the two fish species (inter-specific), *ii)* the individuals of each species (inter-individual), and *iii)* body parts (intra-individual) (KW, *P*>0.05, Fig. 1).

3.2. Beta diversity

Beta diversity between planktonic replicates. Taxonomic composition of planktonic communities presented an important level of variability between water replicates, as shown by Sorensen"s dissimilarity index averaging 0.65 (±0.01, *n*=4) (Fig. 2). When considering phylogenetic proximity between OTUs, dissimilarity dropped by 30% (phyloSor dissimilarity index, 0.46 ±0.01). Values of dissimilarity taking account of relative abundances of OTUs (Shannon beta diversity) or that of phylogenetic lineages (Allen beta diversity) decreased (0.27 ±0.01 and 0.06 ±0.003, respectively). This indicated a strong homogeneity of water replicates when taking into account abundant OTUs and their phylogenetic relatedness.

Beta diversity between water and fish skin. All values of dissimilarity between water and fish skin were high, for each facet and component of diversity considered. Taxonomic compositional and structural beta diversity was almost maximal (Sorensen"s dissimilarity index 0.97 ± 0.03; Shannon beta diversity 0.94 ± 0.07) (Supplementary data S4, Fig. 2). Bacterial dissimilarity between those two habitats decreased by 31% and 45% when we accounted phylogenetic distance alone or associated with OTUs relative abundances (respectively, for phyloSor 0.66 ± 0.05 and Allen beta diversity 0.49 ± 0.1). Nevertheless, whatever the facets and components of diversity considered, the planktonic and fish skin-associated bacterial communities differed significantly (Table 1, PERMANOVA, *P*<0.05).

Beta diversity of skin-associated bacterial communities between fish species. Interspecific dissimilarity was high in terms of taxonomic composition and structure, averaging respectively 0.81 ± 0.03 (Sorensen dissimilarity, S4) and 0.69 ± 0.11 (Shannon beta diversity, Fig. 2). However when considering phylogenetic relationships between lineages, interspecific compositional and structural dissimilarity values halved (phyloSor, 0.43 ± 0.07, S4, and Allen beta diversity, 0.27 ± 0.07, Fig. 2) compared respectively to Sorensen and to Shannon beta diversity. Skin-associated bacterial communities are thus phylogenetically more similar than taxonomically. However, these relatively low interspecific dissimilarities compared to the taxonomic ones were still higher than expected in a null expectation model with hypothesis of no effects of host species effect (Table 1, PERMANOVA, P<0.05).

Beta diversity of skin-associated bacterial community within each species. Inter- and intra-individual dissimilarity values were particularly high, as taxonomic and phylogenetic compositional and structural variability of skin communities for the two fish species was comparable to that observed at the interspecific scale (S4, Fig. 2). However, despite these high differences among individuals and body parts, they were not higher than expected in a null model with hypothesis of no effects of fin type or individual (PERMANOVA, *P*>0.05, Table 1). In the same manner, there was no effect of fish sex on any facet and component of diversity (PERMANOVA, *P*>0.05, Table 1) for each fish species. For each species, skin-associated bacterial communities were therefore variable, and were neither predictable by individuals nor by body parts. However, in each individual, skin-associated bacterial communities differed between fins, as some OTUs were unique to certain fin samples (Fig. 3)

3.3. Dominant phylogenetic groups

Planktonic communities were essentially dominated by the phyla *Proteobacteria* (38 to 54% of sequences obtained from water samples) and *Bacteroidetes* (41 to 52% of sequences) (Fig. 4). Skin communities were mainly composed of members of the phyla *Proteobacteria* (30 to 85% of sequences obtained from fin samples), *Actinobacteria* (2 to 53%), *Bacteroidetes* (0.4 to 27%) and *Firmicutes* (0.4 to 12%) (Fig. 4). *Actinobacteria* and *Firmicutes* lineages were not detected in the planktonic communities. At finer taxonomic levels, there were also disparities between epibiotic and planktonic communities. Planktonic *Proteobacteria* were mainly composed of *Gamma-* and *Alphaproteobacteria*, whereas skin-associated *Proteobacteria* amount to a large fraction of *Betaproteobacteria*. The same disparity was observed for the *Bacteroidetes*, mostly comprised of *Flavobacteria* in planktonic communities, and of *Sphingobacteria* and *Flavobacteria* in skin-associated communities. The two fish species were dominated by the same major bacterial clades (Fig. 4), and the relative abundances of these dominant clades did not significantly differed between the two species (KW, *P*<0.05).

4. Discussion

4.1. Seabass and seabream skin harbored more diverse bacterial communities than water

Skin-associated bacterial communities were particularly diverse, since as many as ca. 73 (±5.4) OTUs were detected on each individual, while only ca. 46 (±2.3) OTUs were detected in 100 mL of water (Fig. 1). Additionally, OTUs abundances were particularly uneven in planktonic communities, with few dominant and a great number of rare ones. Such an uneven distribution of OTU abundance in seawater has been previously reported in the Mediterranean sea by a study using amplicon-based sequencing (Pommier *et al.*, 2010). As

expected, planktonic communities were composed of typical marine classes such as the *Alpha-* and *Gammaproteobacteria* and *Flavobacteria* (Barbern and Casamayor, 2010) (Fig. 4). They likely originated from the marine water that was used to fill up the experimental tanks. In contrast, bacterial OTUs of skin samples had more even abundances distribution than the planktonic communities, and when considering whole individuals, phylogenetic diversity of skin-associated bacterial communities was 50% more diverse than the one of bacterioplankton (Fig. 4). Even distribution of OTUs abundances and high diversity of phylogenetic lineages (Fig 1) of skin-associated bacterial communities could be related to the particular nutritive conditions existing at the fish surface. Most of teleostean fishes secrete mucus, which is constituted by a high diversity of gel-forming glycoproteins, glycosaminoglycans and proteins (Shephard, 1994). Such components can serve as nutrient sources for epibiotic bacteria (Bordas, Balebona, Rodriguez-Maroto, Borrego and Moriñigo, 1998), which thus provide a mix of different resource niches. Environmental complexity and resource partitioning has been shown to favor rich bacterial diversity (Ramette *et al.*, 2007; Schauer *et al.*, 2009). By contrast, in a closed water-circulated system, particles are trapped by a filtration system, potentially inducing particularly homogeneous environmental conditions. In such situation, water may be considered as a desert from a nutritional viewpoint as it provides less nutrients and less nutrient types (Azam and Malfatti, 2007). This may explain the significant phylogenetic clustering observed in water samples. The phylogenetic diversity of skin-associated bacterial communities recorded in our study is greater than that had been evidenced by DNAr 16S cloning and sequencing approach on 6 fish species of the Atlantic ocean, where a total of only 5 different phyla were identified (while as many as 15 phyla were detected on fishes in our study) (Larsen *et al.*, 2013). Such difference could be of methodological order, as clone libraries are known to under-estimate bacterial richness (e.g. comparative study on ant microbiome (Kautz *et al.*, 2013).

Beyond their high diversity, skin bacterial communities exhibited a very different composition from that of their planktonic counterparts, as indicated by the low number of common OTUs (21 OTUs *i.e.* 3% of all detected OTUs, Fig. 3) and high dissimilarity values (S4, Fig. 2) between these two types of habitats, whatever the facets and components of diversity considered. This agrees with recent investigations on wild or outdoor farmed teleostean fishes (Wang *et al.*, 2010; Larsen *et al.*, 2013), although such studies did not permit disentangling intrinsic drivers of microbiome diversity from confounding extrinsic factors (e.g. fish that experienced different water masses due to their mobility and/or change in water masses). Here fishes were raised in controlled conditions during their entire lives, which likely greatly minimizes such artifacts. This line of evidences from different fish species and life histories highlights that the specificity of skin biotope promotes a specific signature of skin bacterial community compared to planktonic cells.

4.2. Phylogenetic homogeneity of skin-associated bacterial communities

The diversity of bacterial epibionts was highly variable across all scales (*i.e.* interspecific, inter- and intra-individual, S4, Fig. 2). However, among the 15 phyla detected on fish skin, more than 95% of OTUs belonged to only 4 clades, namely the phyla *Actinobacteria* and *Firmicutes* and the classes *Sphingobacteria* and *β-proteobacteria* (Fig. 4). This dominance of a core set of phyla drove the low values of phylogenetic structural and compositional dissimilarity (*i.e.* phyloSor and Allen indices, S4 and Fig. 2) compared to taxonomic structural and compositional dissimilarity values. This detected skin bacterial clades are similar to those identified in the gut microbiota of wild and reared *Sparus aurata* and *Dicentrarchus labrax*, but harbored different genera (Carda-Diéguez *et al.*, 2014; Kormas *et al.*, 2014), potentially depicting a specific character of the skin habitat. Interestingly, these skin-associated clades have already been reported in skin microbiota of other vertebrates as teleosts (Wang *et al.*, 2010; Larsen *et al.*, 2013), marine mammals (Apprill *et al.*, 2014), amphibians (Walke *et al.*, 2014), and human (Grice and Segre, 2011). Moreover, a recent

review focusing on marine macroscopic algae and invertebrates surfaces also revealed the same core of high-level bacterial clades (Wahl *et al.*, 2012). To explore such apparent conservatism, we compared the representative sequence of predominant OTUs belonging to each previously cited core phyla and classes with sequences available on Genbank database using BLASTn (http://blast.ncbi.nlm.nih.gov/) (Fig. 5). We observed joint OTUs with other marine vertebrates associated microbiota, water and soil bacterial communities, but the most unforeseen result was that as high as 40% of these fish skin-specific sequences were highly similar (99 to 100% identity, 97 to 100% coverage) with sequences of OTUs previously isolated from healthy human skin surface. These OTUs were identified as *Propionibacterium sp* (representing 1.7-28.8% of sequences in fish samples), followed by *Corynebacterium sp.* (0-9.7%), *Ochrobactrum sp.* (0-9.3%), *Geobacillus sp* (0-8.9%), and *Staphylococcus sp.* (0-7.0%). This similarity between human and fish skin microbiomes was unexpected, as employees of the breeding station were systematically wearing latex gloves for the maintenance of the aquaculture system. In addition, all OTUs belonging to these core clades and recorded in human skin were not detected in water (either because they were absent or sufficiently rare and below the detection threshold, Fig. 5). Shared bacterial species between fish and human is surprising but support the hypothesis that some biotic surface characteristics, which do not exist in the aqueous surrounding environments, may drive skin associated community structure and that these clades may present phylogenetically conserved traits permitting their growth on living surface. Here, further studies are needed to determine whether such similarities at skin surface exist in other animal clades, or if it is a characteristic of teleostean fishes or a result of our particular experimental design. Morevover, for an exhaustive assessment of microbial diversity, such analysis should be extended to viruses, Archaea, and microeukaryotes, as that has been done with human skin microbiome (excepted in the case of viruses) (Findley *et al.*, 2013; Probst *et al.*, 2013).

4.3. Bacterial skin-associated community structure is dependent on fish species

OTU composition in skin-associated bacterial communities differed strongly between the two fish species, as around 70% of OTUs detected at the surface of each species were not detected in the other species (Fig. 3). The high taxonomic structural beta diversity (ca. 70%), which is more informative because it takes into account the relative abundances of OTUs, indicated that among shared species, the bacterial associates of the two fish species were dominated by different OTUs. However the drop of beta diversity values when taking account for phylogenetic affiliation of OTUs (phylogenetic structural beta diversity ca. 30%, Fig 2) demonstrated that, while both species were dominated by different OTUs, these OTUs belonged to phylogenetically close bacterial clades. In addition, this moderate level of phylogenetic structural beta diversity was marked by a significant interspecific difference (Table 1), suggesting that species host close but distinct phylogenetic lineages. Such evidences for host-species specificity were recently observed on 6 teleostean species by Larsen and coworkers (2013). In this study, authors suggested that several physiological species-specific factors (e.g. skin mucous composition, antimicrobial properties) could lead to such pattern. However, as their observations were made from wild animals, it is not easy to partition these effects from others, related to species ecology (*i.e.* mobility, food, contact with biotic and abiotic surfaces), and environmental variations (*i.e.* food availability, physico-chemical conditions of surrounding water masses). Here, hosts were raised under the same conditions (e.g. same food, no antibiotic treatments, same physico-chemical conditions). We therefore can assert that this host-species specificity is primarily due to intrinsic physiologic factors.

Larsen and coworkers (2013) suggested that skin microbiota selection from host species could be considered *i)* as an active selection by the host, or/and *ii)* as a passive selection of bacterial species able to grow on fish surface. Indeed, while general mechanisms of innate immune system are largely conserved among fishes and other vertebrates, the species-

related variability of skin immune components has been proven for fish (Ángeles Esteban, 2012). For example, lysozyme activity, as well as the nature of antimicrobial peptides secreted in the mucus layer, were found to be different between several freshwater fish species (Nigam et al., 2012; Ángeles Esteban, 2012).

In addition, the structure of bacterial communities could be affected by their specific capacities to adhere and grow on skin mucus, as on a culture medium. Fish skin mucus remains poorly documented, yet some species-related variation in mucus characteristics has been reported, such as mucin composition and hydration (*i.e.* mucin concentration) (Roberts and Powell, 2005). Variations in mucus hydration induce changes in its viscoelasticity, which may influence bacterial attachment (Ángeles Esteban, 2012). However, no study to date compared the skin mucus chemical composition between gilthead seabream and European seabass.

4.4. Skin bacterial communities are variable among and within individuals

Among individuals. The high level of inter-individual variability observed (Fig. 2) has already been reported between individuals of wild or *in situ* captive teleosts and cetaceans, and was partly explained by the geographical location (Le Nguyen *et al.*, 2008; Larsen *et al.*, 2013; Apprill *et al.*, 2014). In our study, however, and as discussed before, animals of each species were raised in rigorously same conditions from birth to sampling, which precludes any extrinsic environmental influence on bacterial fish skin associates. This high level of variability between individuals is therefore an intrinsic feature of the studied fish skin bacterial community. Consequently, the effect of geographical location reported above may indeed exceed the intrinsic variability.

Within individuals. The bacterial community composition and structure differed between body parts, as shown by the high values of intra-individual beta diversity (S4 and Fig 2), which was as high as the one between the two fish species. Indeed, 53 to 61% of OTUs detected on each fin were not detected on other ones (Fig. 3), suggesting niche specialization within individuals. However, this intra-individual variability was not explained by fin type when considering the four individuals in each species because of the large inter-individual variability (Table 1, Fig 2). In other words, there is always a difference in bacterial community composition and structure between the four fins in each individual, but when comparing two individuals, a single fin type host different communities. This is in contradiction with studies on the human skin microbiome, which reported that bacterial communities were primarily shaped by skin parts rather than individuals or time (Costello *et al.*, 2009; Grice *et al.*, 2009).

Here the absence of a common community composition pattern between fin types or individuals, coupled with a huge variability at these two scales, suggests that *i)* the body parts studied and individuals did not particularly differ in terms of habitat quality, and therefore *ii)* bacterial communities composition is unpredictable, either because being dependent of stochastic events of bacterial colonization and extinction, or dependent on a large number of interacting factors (e.g. local release of nutrients or antimicrobial molecules). This therefore underlines the need to explore surface-associated communities variability across the entire body, and especially around oral gape, gills and lateral line, which may harbor more contrasted micro-environmental conditions. Further studies are also needed to determine if such pattern exists in other marine or terrestrial vertebrates.

Another possibility is that skin microbiome may temporally vary independently in each individual and body parts. Indeed, a strong temporal variability of skin-associated bacterial communities has been recently reported in human (Costello *et al.*, 2009)., In the case of marine teleosts, studies about temporal dynamics of skin microbiota are very scarce. While a few studies evidenced changes in the composition of cutaneous bacterial communities due

to seasonality and diet changes (Larsen *et al.*, 2013; Landeira-Dabarca *et al.*, 2013), an intrinsic temporal dynamic has never been assessed. Assessing the intra-individual variability of the microbiome for additional fish species and for other aquatic and terrestrial animals is therefore needed to confirm our findings and to identify the drivers of this intra-individual variability.

To conclude, our study demonstrated that fish surface is colonized by a skin-specialized community of bacteria, composed by clades that are not detected in the overlaying water. Skin associates were characterized by a high diversity, which seems to be promoted by their important variability between species, individuals and body parts. As diversity is generally positively correlated to microbial communities functioning and stability (Wittebolle *et al.*, 2009; De Schryver and Vadstein, 2014), and negatively correlated with susceptibility to invaders (De Roy *et al.*, 2013; De Schryver and Vadstein, 2014), this unique biodiversity may favor fish resistance to pathogen invasion through the skin (Wang *et al.*, 2010). Testing the relationship between microbial diversity and infection by pathogens becomes therefore an urgent challenge.

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Conflicts of interest

The authors declare no conflicts of interest.

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Tables

Table 1 : Effect of four factors studied on microbial communities assessed using permutational ANOVAs (PERMANOVA, 999 permutations) on dissimilarity matrices. Bold values indicate a significant effect of the tested factor (P<0.05). For each facet and component of biodiversity, the name of the dissimilarity index is provided in parenthesis

Factor	**Habitat**		**Species**		**Individual**		**Fin**	
Facets & components of biodiversity	*P*	r^2	*P*	r^2	*P*	r^2	*P*	r^2
Taxonomic composition *(Sorensen)*	**0.001**	**0.48**	0.080	0.05	0.353	0.24	0.091	0.12
Phylogenetic composition *(PhyloSor)*	**0.001**	**0.52**	0.114	0.05	0.666	0.22	0.502	0.09
Taxonomic structure *(Shannon)*	**0.001**	**0.48**	**0.01**	**0.06**	0.96	0.18	0.06	0.14
Phylogenetic structure *(Allen)*	**0.001**	**0.54**	**0.01**	**0.11**	0.66	0.26	0.20	0.10

Figures

Fig. 1 : Richness and alpha diversity values of bacterial communities, at all scales (interspecific, inter- and intra-individuals) studied. Points and error bars indicate respectively mean and confidence interval limits (5th and 95th centiles of values obtained from the 1000 bootstraped subsamples) of diversity indices. To facilitate graphs interpretation, taxonomic (*S*, graph (a)) and phylogenetic richness (*PD*, graph (b)) were respectively scaled to the total number of OTUs and the total branch lengths of the phylogenetic tree. (c) Taxonomic structural alpha diversity for each bacterial community, calculated on relative abundance of OTUs using Shannon index. (d) Phylogenetic structural alpha diversity, calculated on relative abundances of terminal branches of the chronogram. On each graph, an asterisk indicates a significant difference of richness and alpha diversity values between water and fin samples (KW, $P<0.05$)

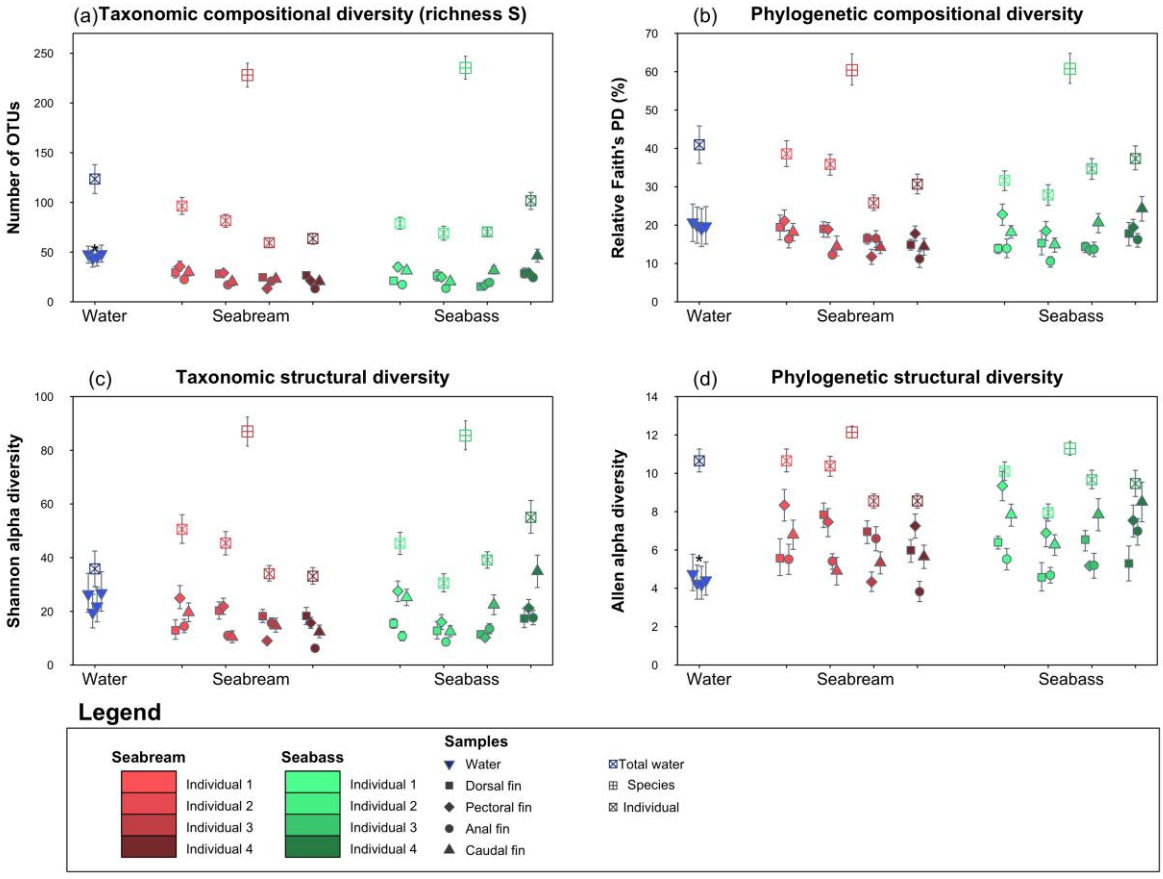


Fig. 2 : Dissimilarity (Beta diversity) among bacterial communities, between individuals for each fin type (“inter-individual”) and between fin types for each individual (“intra-individual”). Total beta diversity within species (“intra-specific”), between species (“interspecific”), between water replicates (“inter-replicate”), and between water and fin samples (“inter-habitat”) are also computed. Taxonomic (a) and phylogenetic (b) structural beta diversities were computed using the multiplicative decomposition of the Shannon and the Allen indices, respectively. Squares represent mean beta diversity values at the considered scale, and error bars indicate standard deviation of the *n* diversity values (at the center of each square) used to calculate the mean. Different letters indicate significant differences between beta diversity values (KW, $P<0.05$)

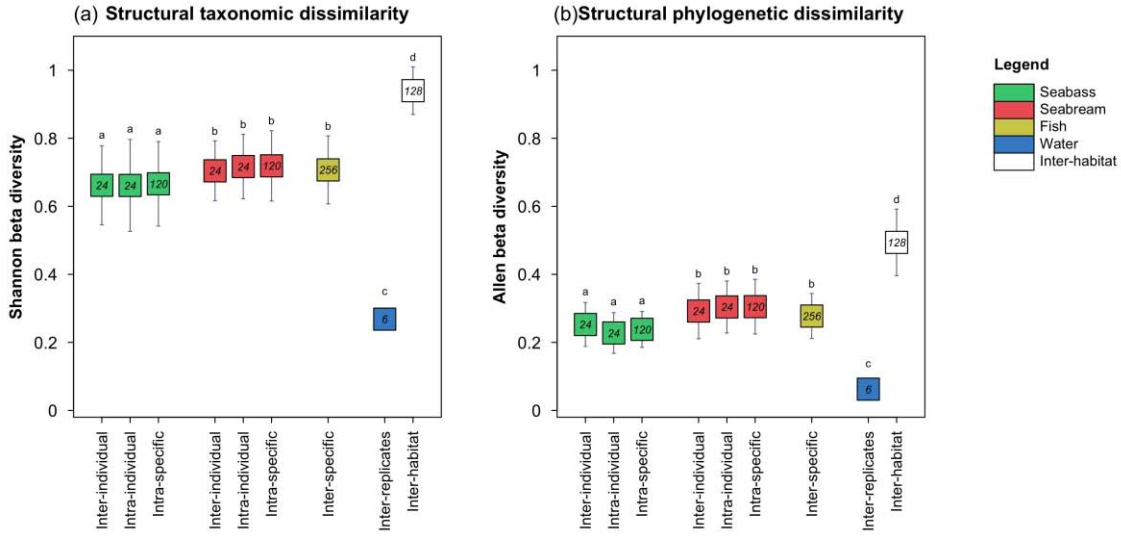


Fig. 3 : Venn diagrams of detected OTUs (a) on skin surface of the two fish species and in water samples, and (b) on each fish fin type, summed with all individuals of the two fish species. Venn diagramm (a) is scaled to actual numbers of OTUs and overlaps. Venn diagramm (b) is not proportional to numbers of OTUs and overlaps.

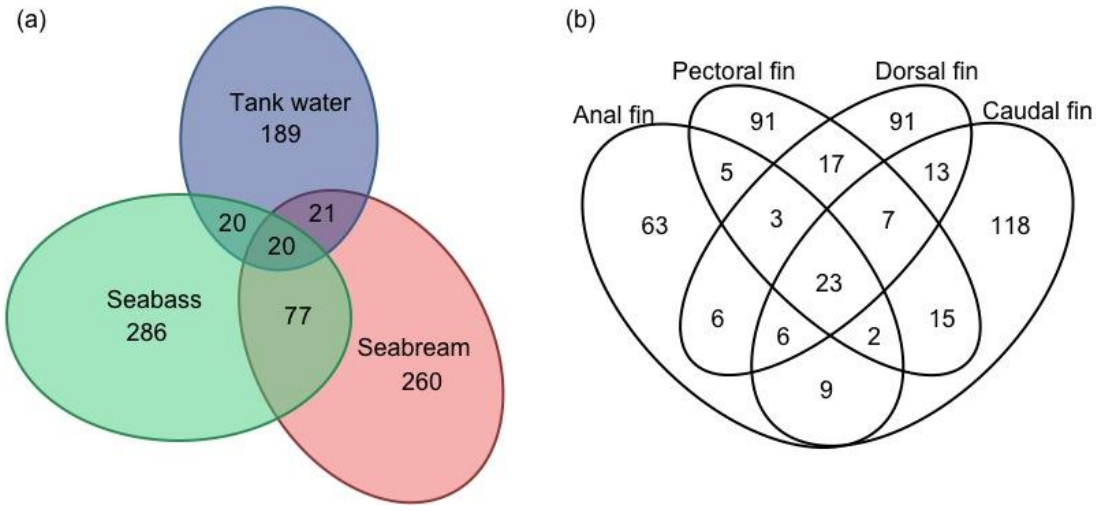


Fig. 4 : Relative abundances of main bacterial phyla and classes in each sample. Bacterial Sequences that couldn't be classified at the phylum level with a confidence threshold of 80% were depicted as "Unidentified"

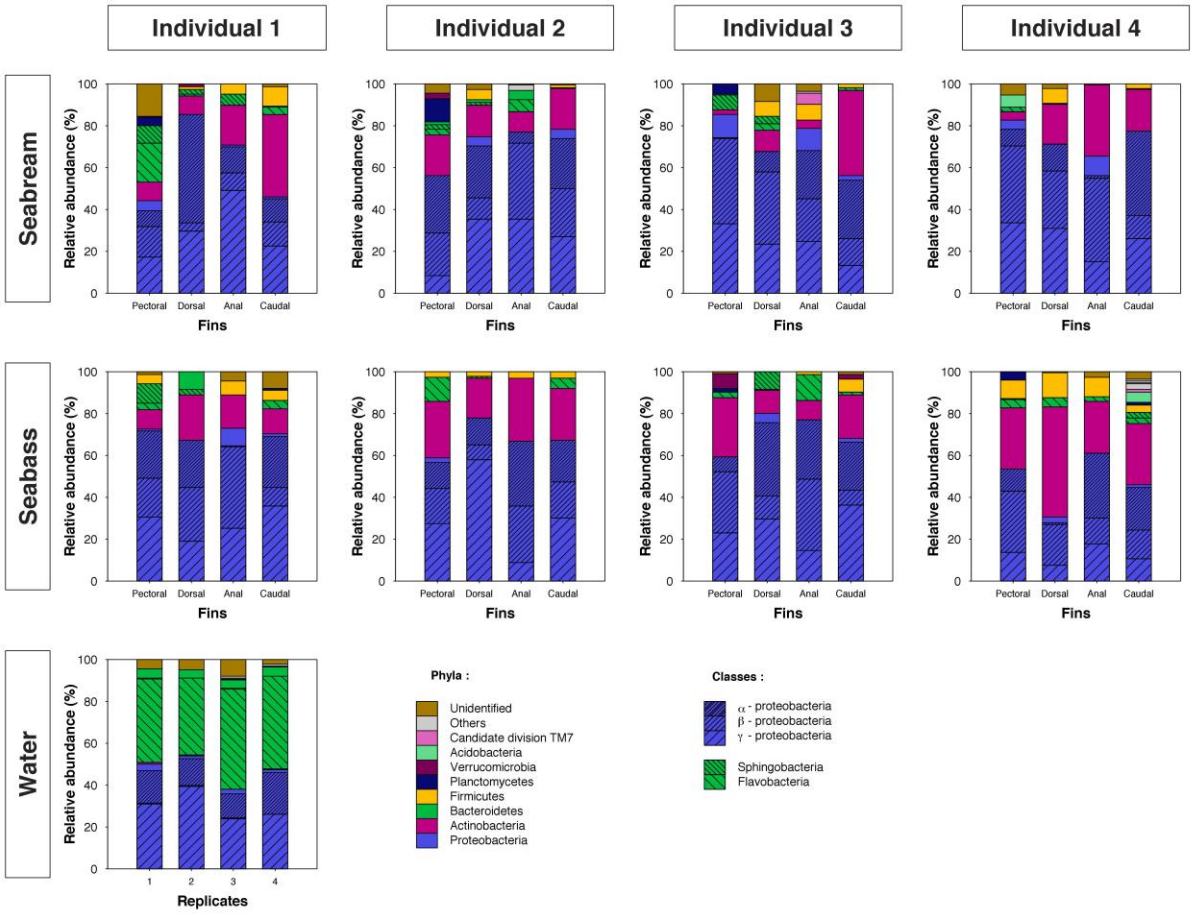
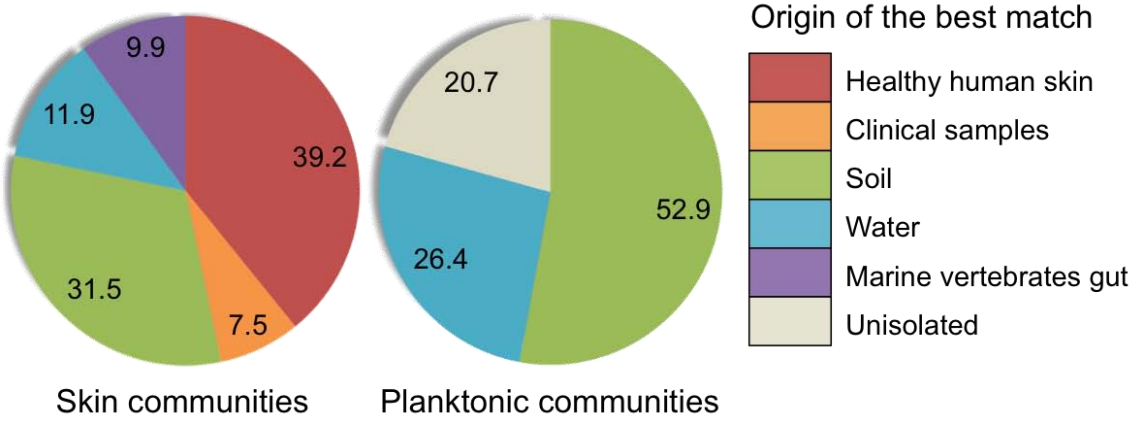


Fig. 5 : Results of GenBank search upon the 50 most abundant OTUs of main phyla recovered in skin samples. For each OTU, the representative sequence was compared to Genbank sequences using BLASTn. For each sequence, the best match (≥97% coverage, ≥99% identity) was selected, and the origin of the samples was noted. OTUs with no good matches were grouped in the "Unisolated" class



Supplementary data S1 : Physico chemical conditions at the time of sampling, measured using a multiparameter probe (YSI Professional Plus, Yellow Springs Instrument, Ohio. USA).

	Seabream tank	**Seabass tank**
Temperature (°C)	19.6	19.6
Salinity (PSU)	37.3	37.3
pH	7.52	7.47
Conductivity ($mS.cm^{-1}$)	50.2	50.3
Dissolved Oxygen (%)	75.5	64
Pressure (mm Hg)	754.9	755
Redox potential (mV)	-19.3	-17.6

Sample Number	Species	Individual	Skin site	Number of sequences
Sample10	Seabass	1	Dorsal fin	234
Sample12	Seabass	2	Dorsal fin	1745
Sample14	Seabass	3	Dorsal fin	514
Sample16	Seabass	4	Dorsal fin	1352
Sample18	Seabass	1	Pectoral fin	789
Sample187	Water	NA*	NA	11446
Sample188	Water	NA	NA	11535
Sample192	Water	NA	NA	11952
Sample193	Water	NA	NA	10780
Sample20	Seabass	2	Pectoral fin	400
Sample22	Seabass	3	Pectoral fin	113
Sample24	Seabass	4	Pectoral fin	789
Sample26	Seabass	1	Anal fin	536
Sample28	Seabass	2	Anal fin	556
Sample30	Seabass	3	Anal fin	429
Sample32	Seabass	4	Anal fin	578
Sample34	Seabass	1	Caudal fin	1262
Sample36	Seabass	2	Caudal fin	396
Sample38	Seabass	3	Caudal fin	1469
Sample40	Seabass	4	Caudal fin	903
Sample51	Seabream	1	Dorsal fin	3131
Sample52	Seabream	2	Dorsal fin	479
Sample53	Seabream	3	Dorsal fin	504
Sample54	Seabream	4	Dorsal fin	816
Sample59	Seabream	1	Pectoral fin	995
Sample60	Seabream	2	Pectoral fin	882
Sample61	Seabream	3	Pectoral fin	325
Sample62	Seabream	4	Pectoral fin	376
Sample67	Seabream	1	Anal fin	1190
Sample68	Seabream	2	Anal fin	223
Sample69	Seabream	3	Anal fin	391
Sample70	Seabream	4	Anal fin	1156
Sample75	Seabream	1	Caudal fin	788
Sample76	Seabream	2	Caudal fin	935
Sample77	Seabream	3	Caudal fin	709
Sample78	Seabream	4	Caudal fin	1066
			Total	**71744**

Number of sequences recovered in each sample after Mothur pipeline

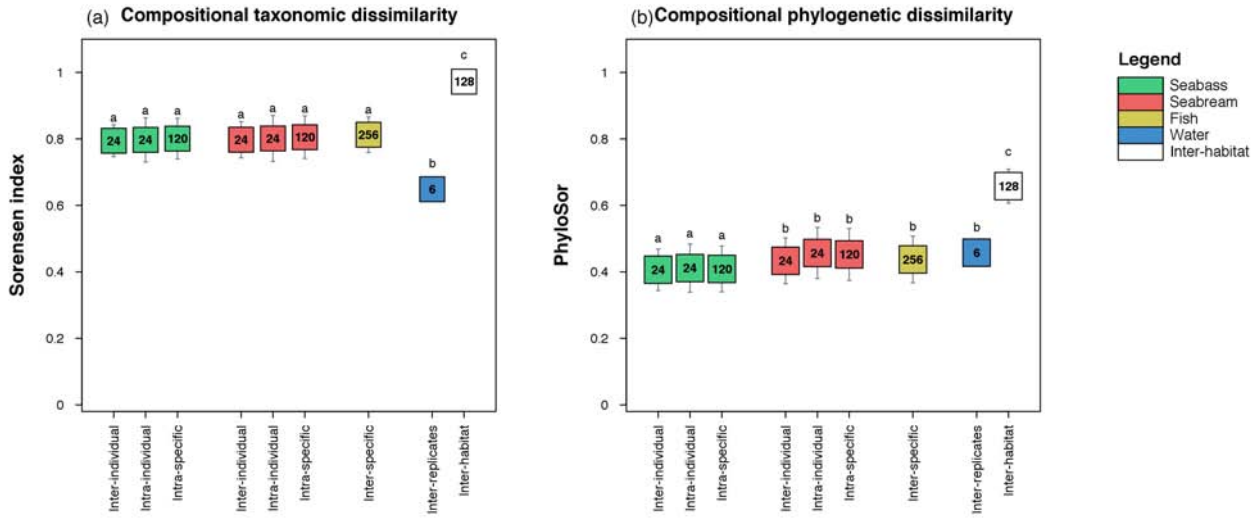
*NA : Not Applicable

Supplementary data S2: List of calibration points used for the chronogram fitting step, in order to obtain a dated ultrametric tree grouping all defined OTUs. Clades were chosen to cover most of phylogenetic tree branches at various tree depths. Divergence times were obtained from synthetic work of Hedges et al [1. 2].

Bacterial clades		**Divergence times (Ma)**	
Clade A	Clade B	Minimum	Maximum
Archaea	*Bacteria*	4200	4200
Planctomycetes	*Bacteroidetes*	2747	3040
Actinobacteria	*Firmicutes*	2755	3041
Burkholderiaceae	*Alcaligenaceae*	640	859
Pseudomonadaceae	*Moraxellaceae*	1190	1420
Alteromonadales	*Vibrionales*	849	1051
Bacillaceae	*Clostridiaceae*	2448	2983
Deinococcus-Thermus	*Proteobacteria*	2850	3186

1. Hedges SB, Dudley J, Kumar S (2006) TimeTree: a public knowledge-base of divergence times among organisms. Bioinformatics 22:2971–2972. doi: 10.1093/bioinformatics/btl505

2. Hedges SB, Kumar S (2009) The Timetree of Life. Oxford University Press



Supplementary data S4: Compositional dissimilarity (Beta diversity) among bacterial communities, between individuals for each fin type ("inter-individual"), between fin types for each individual ("intra-individual"). Total beta diversity within species ("intra-specific"), between species ("interspecific"), between water replicates ("inter-replicate"), and between water and fin samples ("inter-habitat") are also computed. (a) Taxonomic and (b) Phylogenetic compositional beta diversities, were computed using the Sorensen and the phyloSor indices, respectively. Squares represent mean beta diversity values at the considered scale, and error bars indicate standard deviation of the *n* diversity values (at the center of each square) used to calculate the mean. Different letters indicate significant differences between beta diversity values (KW, $P<0.05$)