RESEARCH ARTICLE

Diversity of the skin microbiota of fishes: evidence for host species specificity

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Abstract

Skin microbiota of Gulf of Mexico fishes were investigated by ribosomal internal spacer analysis (RISA) and 16S rRNA gene sequencing. A total of 102 fish specimens representing six species (*Mugil cephalus*, *Lutjanus campechanus*, *Cynoscion nebulosus*, *Cynoscion arenarius*, *Micropogonias undulatus*, and *Lagodon rhomboides*) were sampled at regular intervals throughout a year. The skin microbiota from each individual fish was analyzed by RISA and produced complex profiles with 23 bands on average. Similarities between RISA profiles ranged from 97.5% to 4.0%. At 70% similarity, 11 clusters were defined, each grouping individuals from the same fish species. Multidimensional scaling and analysis of similarity correlated the RISA-defined clusters with geographic locality, date, and fish species. Global *R* values indicated that fish species was the most indicative variable for group separation. Analysis of 16S rRNA gene sequences (from pooled samples of 10 individual fish for each fish species) showed that the *Proteobacteria* was the predominant phylum in skin microbiota, followed by the *Firmicutes* and the *Actinobacteria*. The distribution and abundance of bacterial sequences were different among all species analyzed. *Aeribacillus* was found in all fish species representing 19% of all clones sequenced, while some genera were fish species-specific (*Neorickettsia* in *M. cephalus* and *Microbacterium* in *L. campechanus*). Our data provide evidence for the existence of specific skin microbiota associated with particular fish species.

Introduction

Microbiota refers to the community of microorganisms occupying a specific ecosystem. Focused primarily on human gut microbiota, recent studies have promulgated a new microbiologic paradigm that posits a 'healthy' or 'normal' microbiota is critical to human health (Simpson *et al.*, 2005; Mueller *et al.*, 2012). Such a 'healthy microbiota' concept applies to the bacteria associated with any tissue that has an epithelial membrane, for example, epidermis and olfactory, respiratory, and urogenital mucosas (Costello *et al.*, 2009; Grice *et al.*, 2009; Robinson *et al.*, 2010; Ravel *et al.*, 2011). In humans, the maintenance of 'healthy' microbiota reportedly is linked with the prevention of infectious diseases, production of amino acids and vitamins essential for host homeostasis, and lower predisposition to diabetes, allergies and, in some instances, cancers (Robinson *et al.*, 2010). Such host–bacteria mutualism has been documented in mammal models such as the mouse (Turnbaugh *et al.*, 2006), domesticated animals, including chicken (Barnes, 1972), pig (Leser *et al.*, 2002), and cow (Brulc *et al.*, 2009), insects (Ohkuma, 2008), and marine invertebrates (Duperron *et al.*, 2005; Di Camillo *et al.*, 2012).

The largely ectothermic aquatic vertebrates (=fishes), which comprise the majority of vertebrate species (Nelson, 2006), and their associated microbiota are vastly underexplored. Specifically, scant information exists on the biodiversity, geographic distribution, and seasonality of microbiota on marine, estuarine, and freshwater fishes. The majority of published studies treating fish microbiota focus on gut microbiota. Most of these studies experimentally modified and/or supplemented diets with probiotics toward promoting a gut microbiota optimal for fish growth and disease resistance (Hjelm *et al.*, 2004; Schulze *et al.*, 2006; Fjellheim *et al.*, 2008; Gatesoupe, 2008;

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Gomez & Balcazar, 2008). Mouchet *et al.* (2011) characterized the genetic diversity of the gut microbiota associated with 15 fish species of the southwestern Atlantic Ocean off Brazil and showed that the genetic diversity of the fish gut microbiota was significantly influenced by geographic locality, diet, and fish species, while the functional diversity was mainly determined by diet and fish species. The microbiota present on the skin of fishes are far less studied, but do show some level of specificity (Smith *et al.*, 2007; Wilson *et al.*, 2008). Some have been used as biologic tags indicating where fish were originally captured from the wild (Smith *et al.*, 2009) and where they were cultured prior to being processed and packaged for market (Nguyen *et al.*, 2008). Seasonal shifts in the composition of fish skin microbiota are known in wild Atlantic cod (*Gadus morhua*; Wilson *et al.*, 2008) and aquacultured catfish (*Pangasius* sp.; Nguyen *et al.*, 2008).

The evolutionary origins and ancestry of fish microbiota remain largely unstudied, and, as a result, whether or not fish species harbor unique microbiota is poorly understood. Horsley (1973) used culture-based methods to conclude that microbiota of fish epidermis and mucus were representative of whichever bacteria occurred in the fish's water. However, culture-based surveys vastly underestimate microbiota diversity because an estimated < 10% of bacteria can be isolated and cultured under laboratory conditions (Amann *et al.*, 1995). Nevertheless, some of these pioneering studies surveyed the microbiota of various fishes (Georgala, 1953; Colwell & Liston, 1962; Horsley, 1973, 1977) and identified seasonal and biogeographic patterns of variation similar to those revealed by culture-independent methods (Wilson *et al.*, 2008). None of these studies supported a strong correlation between a fish species and a unique microbiota; however, few compared microbiota across fish species (Colwell & Liston, 1962). That bacteria can seemingly benefit their hosts, or persist as commensals on the surface of their fish hosts, and potentially show some level of specificity to certain fish lineages could together support the notion of a long-standing symbiosis. Perhaps these relationships have existed long enough to exhibit cophyly. Epidermis and mucus of fish constitute an immunologically active and dynamic barrier that prevents pathogen colonization and subsequent infections that may result in a disease condition. Therefore, it seems logical to hypothesize that the microorganisms of the fish skin microbiota have established a close relationship with their host, similar to those that have colonized the nasopharyngeal cavity in humans and other vertebrates (Bogaert *et al.*, 2011). These hypotheses remain largely untested using modern molecular approaches, principally due to a lack of foundational descriptive information on the species identities (community composition) of those bacteria that form the microbiota of fishes.

To that end, the objective of this study was to apply culture-independent methods to characterize and compare microbiota on skin of several teleostean fishes, that is, striped mullet (*Mugil cephalus*; *Mugiliformes*: *Mugilidae*), red snapper (*Lutjanus campechanus*; *Perciformes*: *Lutjanidae*), spotted seatrout (*Cynoscion nebulosus*; *Perciformes*: *Sciaenidae*), sand seatrout (*Cynoscion arenarius*), Atlantic croaker (*Micropogonias undulatus*; *Perciformes*: *Sciaenidae*), and pinfish (*Lagodon rhomboides*; *Perciformes*: *Sparidae*), of the north-central Gulf of Mexico. Based on previous studies, we hypothesized that season (temperature) will be the primary force shaping the diversity and structure of fish skin microbiota.

Materials and methods

Collections

Sampling began in June 2010 and continued monthly through December 2010 with one additional sampling during September 2011. Sampling locations including coastal waters of Dauphin Island (DI; 30°14′55″N 88°04′29″W) and Orange Beach (OB; 30°14′50″W 87°40′01″W) in Alabama and Ocean Springs (OS; 30°23′31″N 88°47′54″W) in Mississippi. The offshore site (28°57′20″N 89°44′37″W) was *c.* 30 km west of the mouth of the Mississippi River in Louisiana (LA). Table 1 summarizes collection dates, locations, and numbers of fish analyzed per collection event. One litre of seawater was collected at each location using a sterile container (except for the offshore location). Seawater surface temperature was measured at 1 m depth *in situ* using a mercury-in-glass thermometer (SargentWelch). Salinities were measured with a handheld refractometer (Vital Sine™ Model SR-6). Fishing efforts lasted between 4 and 8 h, except for the offshore location wherein fish were collected as part of a 3-day fisheries research cruise. Fish were captured using standard baited hooks and 20 (100 for red snapper) pound test monofilament fishing line on spinning reels. Hooked fish were raised from the water, secured and suspended in air by the angler grasping the leader base or hook shaft, and then touched only by a second worker wearing sterile surgical gloves and equipped with flamed and ethanol-rinsed, heavy-gauge scissors. In coordination with raising the fish from the water, the second worker approached and immediately excised a portion (*c.* 1 cm^2) of the dorsal fin. The tissue was placed in a sterile 1.7-mL centrifuge tube and frozen at −20 °C until further processing. Species sampled were striped mullet (*M. cephalus*; *Mugiliformes*: *Mugilidae*), red snapper (*L. campechanus*; *Perciformes*: *Lutjanidae*), spotted seatrout (*C. nebulosus*; *Perciformes*: *Sciaenidae*), sand seatrout (*C. arenarius*), Atlantic croaker (*M. undulatus*; *Sciaenidae*), and pinfish (*L. rhomboides*; *Perciformes*: *Sparidae*).

Table 1. Temporal and spatial distribution of fishing efforts summarizing number of fish analyzed in the study

Fish species (Order: Family), common name	No. of fish	Locality	Date
Mugil cephalus	1	OS	July 2010
(*Mugiliformes*:	14	OS	December 2010
Mugilidae), striped mullet			
Total	15		
Lutjanus campechanus	25	LA	September 2011
(*Perciformes*: *Lutjanidae*),			
red snapper			
Total			
Lagodon rhomboides	2	DI	August 2010
(*Perciformes*: *Sparidae*),	2	DI	September 2011
pinfish	1	DI	October 2010
	3	DI	November 2010
	1	MB	June 2010
	2	OS	July 2010
	1	OS	September 2010
	5	OB	October 2010
Total	17		
Cynoscion arenarius	1	MB	June 2010
(*Perciformes*: *Sciaenidae*),	9	OS	July 2010
sand seatrout	6	OS	September 2010
	8	OS	November 2012
Total	24		
Cynoscion nebulosus	1	DI	August 2010
(*Perciformes*: *Sciaenidae*),	9	MB	June 2010
spotted seatrout	1	OS	December 2010
Total	11		
Micropogonias undulatus	7	DI	August 2010
(*Perciformes*: *Sciaenidae*),	1	DI	September 2010
Atlantic croaker	9	MB	June 2010
	1	OS	July 2010
	3	OS	September 2010
	6	OS	December 2010
Total	27		
Total fish sampled	102		

OS, Ocean Springs, MS; LA, offshore of Grand Isle, LA; DI, Dauphin Island, AL; MB, Mobile Bay, AL; OB, Orange Beach, AL.

All fish were identified according to Carpenter (2001). Ordinal and familial classifications of fishes follow Nelson (2006). Common names for fishes follow Eschmeyer (2010).

DNA extraction and PCR

The DNeasy Blood & Tissue kit (Qiagen, Valencia, CA) was used for fish DNA extractions following manufacturer's instructions with the following adaptations: To ensure extraction from Gram-positive bacteria, a treatment with lysozyme was incorporated as the first step in the protocol, followed by a proteinase K treatment that lasted for 15 h, and DNA was eluted twice with 50 μL elution buffer. Water samples were centrifuged at 10 000 ***g*** for 20 min. Supernatants were discarded, and DNA was extracted from pellets using the protocol described above. Extracted DNA was used as a template for PCR on the internal transcribed spacer region using the ITS-FEub (5′-GTCGTAACAAGGTAGCCGTA-3′) and ITS-REub (5′-GCCAAGGCATCCACC-3′) primers (Cardinale *et al.*, 2004). Ribosomal internal spacer analysis (RISA) was performed as previously described by Arias *et al.* (2006) with the following modifications. The PCR mix contained 1× Taq buffer, 0.4 mM dNTPs (Promega, Madison, WI), 0.4 μM ITS-FEub primer, 0.2 μM ITS-R primer, 0.02 μM ITS-REub labeled primer, 5 mM $MgCl_2$, 1 U of Taq polymerase (5 PRIME, Inc., Gaithersburg, MD), and 100 ng of template DNA in a final volume of 50 μL. PCR conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 45 s, 55 °C for 1 min, and 68 °C for 2 min, ending with a final extension at 68 °C for 7 min. For water samples, a second round of PCR (as per above) was needed to visualize the RISA bands. Ten microliters of each PCR product was diluted with 5 μL AFLP® Blue Stop Solution (LI-COR). Diluted samples were denatured at 95 °C for 5 min followed by rapid cooling prior to gel loading to prevent reannealing. PCR products were electrophoresed on the NEN Global Edition IR2 DNA Analyzer (LI-COR) following manufacturer's instructions. One microliter of sample was loaded into each well.

Sequencing

To identify the predominant bacterial species on the fish skin, we used a 'shot-gun sequencing' approach using DNA extracted from selected individual fish. Equimolecular amounts of DNAs from 10 individuals from the same species were mixed, and the 16S rRNA gene was amplified by PCR. In short, 16S rRNA gene amplification was performed using the universal primers Bact-8F (5′-AGA GTTTGATCCTGGCTCAG-3′) and UNI534R (5′-ATTAC CGC GGCTGCTGG-3′) that amplified the variable regions V1–V3. PCR reagents and conditions have been described elsewhere (Edwards *et al.*, 1989; Muyzer *et al.*, 1993). Purified amplified products were cloned into the pCR-4-TOPO vector and transformed into competent *Escherichia coli* One Shot TOP10 using the TOPO-TA cloning kit for sequencing (Invitrogen, San Diego, CA). Ninety-six clones were randomly selected from each fish species. Clones were automatically sequenced using an ABI 3730xl sequencer at Lucigen Corp. (Madison, WI).

Data analyses

RISA images were processed with BioNumerics v. 6.6 (Applied Maths, Austin, TX). Following conversion, normalization, and background subtraction with mathematical

algorithms, levels of similarity between fingerprints were calculated with the Pearson product-moment correlation coefficient (r). Cluster analysis was performed according to Arias *et al.* (2006) using the unweighted pair-group method with arithmetic mean (UPGMA). Multidimensional scaling (MDS) was performed using optimized positions. Analysis of similarities (ANOSIM) was run from the similarity matrix generated in BioNumerics using Primer v6 (Primer-E Ltd, Plymouth, UK). DNA sequences were read and edited by the software Chromas Version 1.45 (Conor McCarthy, School of Health Science, Griffith University, Gold Coast campus, Southport, Qld, Australia) and loaded into the Ribosomal Database Project (RDP). The classifier tool was used to identify bacteria to the genus level (Wang *et al.*, 2007). Sequencing results were grouped taxonomically at the phylum level. Data were analyzed using SIMPER analysis in Primer v6.

Results

Fish captured

A total of 102 fish specimens representing six species, five genera, four families, and two orders were sampled at regular intervals (Table 1). While red snappers were only captured in Louisiana waters during the month of September 2011 and striped mullets only in Ocean Springs, MS, specimens of the other species were caught during a variety of months at multiple locations wherein temperatures and salinities were 16−32 °C and 8−27‰, respectively (Table 2).

Individual fish external microbiota

The skin microbiota of each fish was fingerprinted by RISA. Each RISA profile consisted on average of 23 bands ranging from 50 to 700 bp. Figure 1 shows a typical RISA profile from each of the fish species analyzed. After creating a similarity matrix based on pairwise comparisons using the Pearson product-moment correlation coefficient, a dendrogram was derived by UPGMA clustering analysis (Fig. 2). The similarity between individual fish skin microbiota ranged from 97.5% to a minimum of 4.0%. In Fig. 2, branches grouping profiles with $\geq$ 70% similarity from the same fish species were collapsed for ease of viewing. The cutoff point of 70% was chosen based on the reproducibility and repeatability of the RISA technique under our conditions. Previous studies from our group showed that up to 25–30% of the dissimilarity observed among RISA profiles can be due to variability introduced by the method (Arias *et al.*, 2006). At 70% similarity, 11 clusters representing three or more individual fish were defined. All 11 of those clusters grouped individual fish from the same species. These clusters contained a total of 52.9% of all individuals sampled. Seven clusters contained only one sampling month, representing 31.1% of the samples. Seven clusters also contained only one sampling location. These clusters included 26.9% of the individuals sampled. Seawater microbiota were also analyzed by RISA. However, the low amount of DNA obtained after extraction required two rounds of PCR amplification before the RISA profiles could be visualized. Therefore, side-by-side comparison of seawater samples along with fish samples was not possible. The clustering analysis of the RISA seawater samples is shown in Supporting Information, Fig. S1. No clear correlation between sampling location or date and percent of similarity between seawater samples could be inferred.

Table 2. Water temperature and salinity of collection sites

Date	Location	Temperature (°C)	Salinity (‰)
June 2010	Mobile Bay, AL	32	12
July 2010	Ocean Springs, MS	30	8
August 2010	Dauphin Island, AL	30	27
September 2010	Dauphin Island, AL	30	24
	Ocean Springs, MS	30	nd
October 2010	Dauphin Island, AL	23	29
	Orange Beach, AL	22	20
November 2010	Dauphin Island, AL	18	27
	Ocean Springs, MS	20	17
December 2010	Ocean Springs, MS	16	17
September 2011	Offshore, LA	27	nd

nd, not determined.

Variables affecting microbiota structure

MDS was utilized to better visualize the groups defined by the RISA-based clustering analysis. Skin microbiota profiles were ascribed to groups based on the variables analyzed (date, location, and fish species), and their RISA similarities were represented by MDS plots. Figure 3 shows the MDS plots when fish species was used as a variable. ANOSIM was used to test the significance of the groupings for each variable. This analysis indicates the significance of groups based on a given factor; in this case, we analyzed fish species, sampling date, and location. The results from the ANOSIM indicate whether the samples are statistically separated by a factor (significance at $P < 0.05$) and the extent of separation (given as a global R-value). If $P < 0.05$, the samples significantly grouped by the tested factor. Higher R-values indicate less overlap in samples, or greater group separation. Thus, both the P-value and R-value must be interpreted to understand the extent to which a factor influences group separation (Clarke & Gorley, 2006). In general, if an

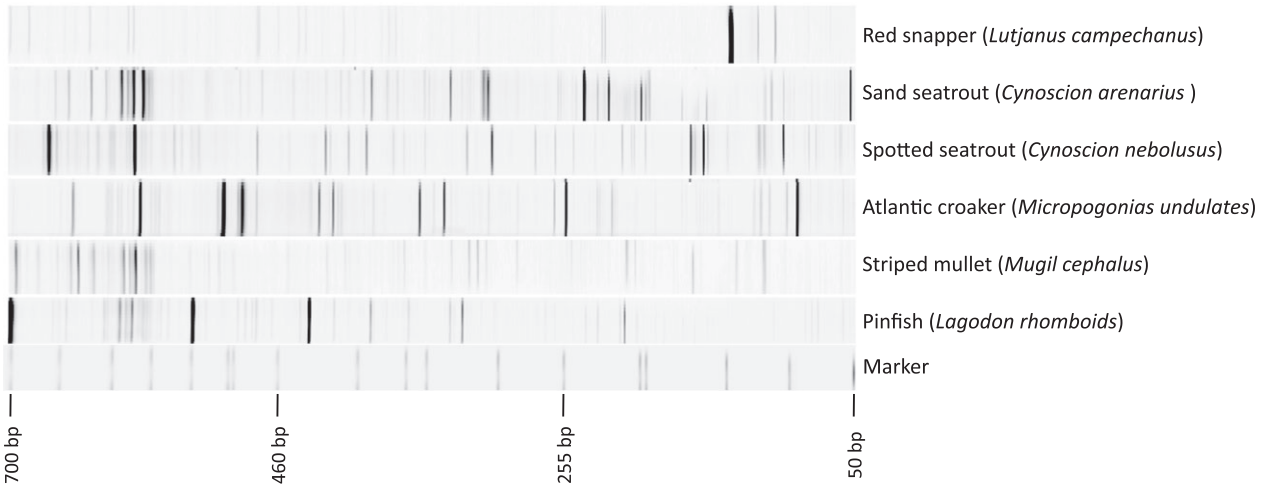

Fig. 1. RISA profiles obtained from one individual of each fish species. Molecular weight marker indicates size range of the RISA profiles.

R-value is < 0.25, the groups have little separation, if it is > 0.5, there is some overlap but the groups are separated, and if the value is > 0.75, there is large separation between groups. Each variable was found to be significant ($P = 0.001$), and global *R*-values were above 0.25 in all cases, ranging from 0.338 to 0.549 (Table 3).

When skin microbiota were grouped by date, data showed that samples collected at different months differed from each other except in two cases. Microbiota from fish collected in September 2010 were not statistically different from those collected in July, October, and November 2010. Similarly, samples from September 2011 could not be separated from those collected in October 2010. The global *R* value for date was 0.338 with pairwise comparison *R* values ranging from 0.189 to 0.635. When samples were assigned to groups based on location, all sites were found to be statistically different with the exception of Orange Beach, which could not be separated from the other locations. The global *R*-value for location ($R = 0.362$) was similar to the global *R*-value for date as were the pairwise *R*-values associated with date group comparisons (*R*-values from 0.116 to 0.568).

Conversely, when groups were assigned based on fish species, the global *R*-value was much higher ($R = 0.549$), indicating that fish species was the most indicative variable for group separation. Pairwise comparisons (not shown) indicate that each fish species group was significantly separated from each other group ($P < 0.05$) with *R*-values ranging from 0.330 to 0.848.

Dominant microbiota

As fish species showed the highest significance for grouping the microbiota, 10 individuals from each species were pooled together for sequencing. In order to obtain maximum bacterial diversity present within a fish species, representatives for each species that were scattered throughout the dendrogram were selected for analysis. Two plates of 96 samples each were sequenced for each fish species. However, we only obtained 69 high-quality sequences (> 400 bp) from spotted trout; thus, we normalized the number of sequences to be compared by randomly selecting 69 sequences from each species. Sequences were identified at the genus level using the classifier tool of the RDP database. All sequences have been deposited in GenBank under the accession numbers (JX543531–JX543948).

When sequences were ascribed at the phylum level, each fish species returned a unique distribution of bacteria (Fig. 4). The *Proteobacteria* was the predominant phylum and represented at least 42% of sequences from each fish species and 61% of all identified sequences. The second most predominant phylum in all fishes was the *Firmicutes*, with species of that phylum comprising 13–42% of the skin microbiota. *Actinobacteria*, *Bacteroidetes*, and *Cyanobacteria* were also identified, constituting 6%, 4%, and 1% of all sequences, respectively. SIMPER analysis indicated that, based on phylum composition, pinfish and spotted seatrout were the most similar (89.8%), while sand seatrout and red snapper were the least similar (61%). Red snapper was the least similar on average to all other fish species (67.5% similarity), while spotted seatrout was on average the most similar to all other fish species (78.8% similarity).

In all species but striped mullet, members of the *Gammaproteobacteria* class constituted about 50% of all the *Proteobacteria* followed in abundance by *Betaproteobacteria*. Striped mullet presented a different composition

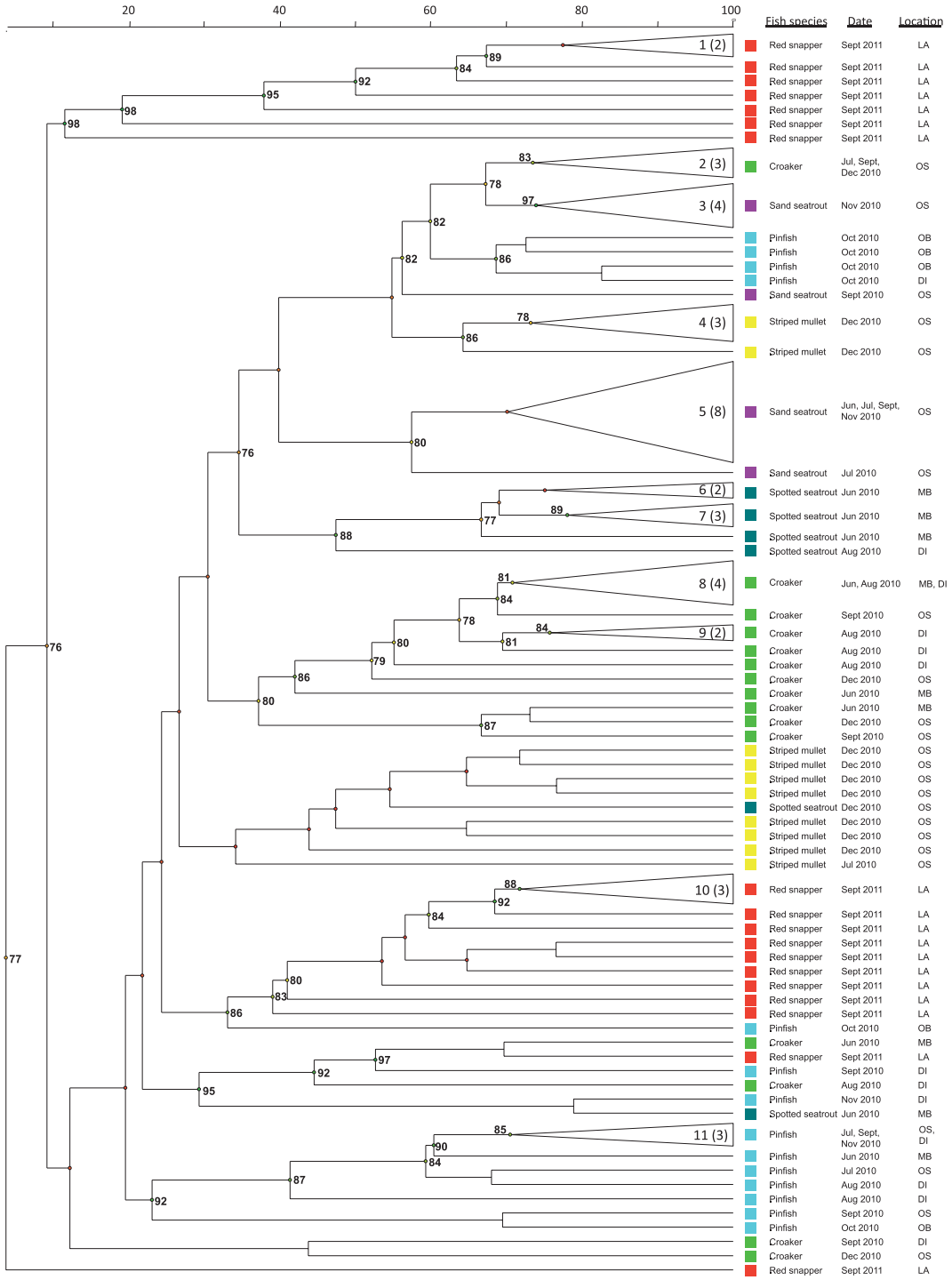

Fig. 2. RISA patterns obtained from individual fish analyzed in the study. Fish species, location, and date for each fish are specified. The scale represents the percent of similarity using the Pearson product-moment correlation coefficient. The dendrogram was constructed using UPGMA. Clusters were defined at 70% similarity; number of individual fish per cluster is shown in parentheses. Cophenetic correlation coefficients, reflecting the robustness of each node are indicated (only values over 75% are shown).

of classes of the *Proteobacteria*, with the *Alphaproteobacteria* being the dominant group followed by the *Gammaproteobacteria* and *Betaproteobacteria*. Atlantic croaker and spotted seatrout showed the least diverse *Proteobacteria* group with representatives of only the *Gamma-* and *Betaproteobacteria* (Fig. 4).

Table 3. Analysis of similarity values obtained when skin microbiota profiles were ascribed to spatiotemporal variables and to host species

Group	Global *R*	Significance	Permutation ≥ Global *R*
Date	0.338	0.001	0
Location	0.362	0.001	0
Species	0.549	0.001	0

Figure 5 illustrates the most common (> 5 representative sequences from at least one fish species) bacterial genera associated with all fish species. *Aeribacillus* was abundant on all fish species and accounted for 19% of all sequenced clones. *Pseudomonas* was identified from all fish species except Atlantic croaker and represented 11% of all clones sequenced. *Janthinobacterium* was the third most frequently identified genus (10%) but absent on red

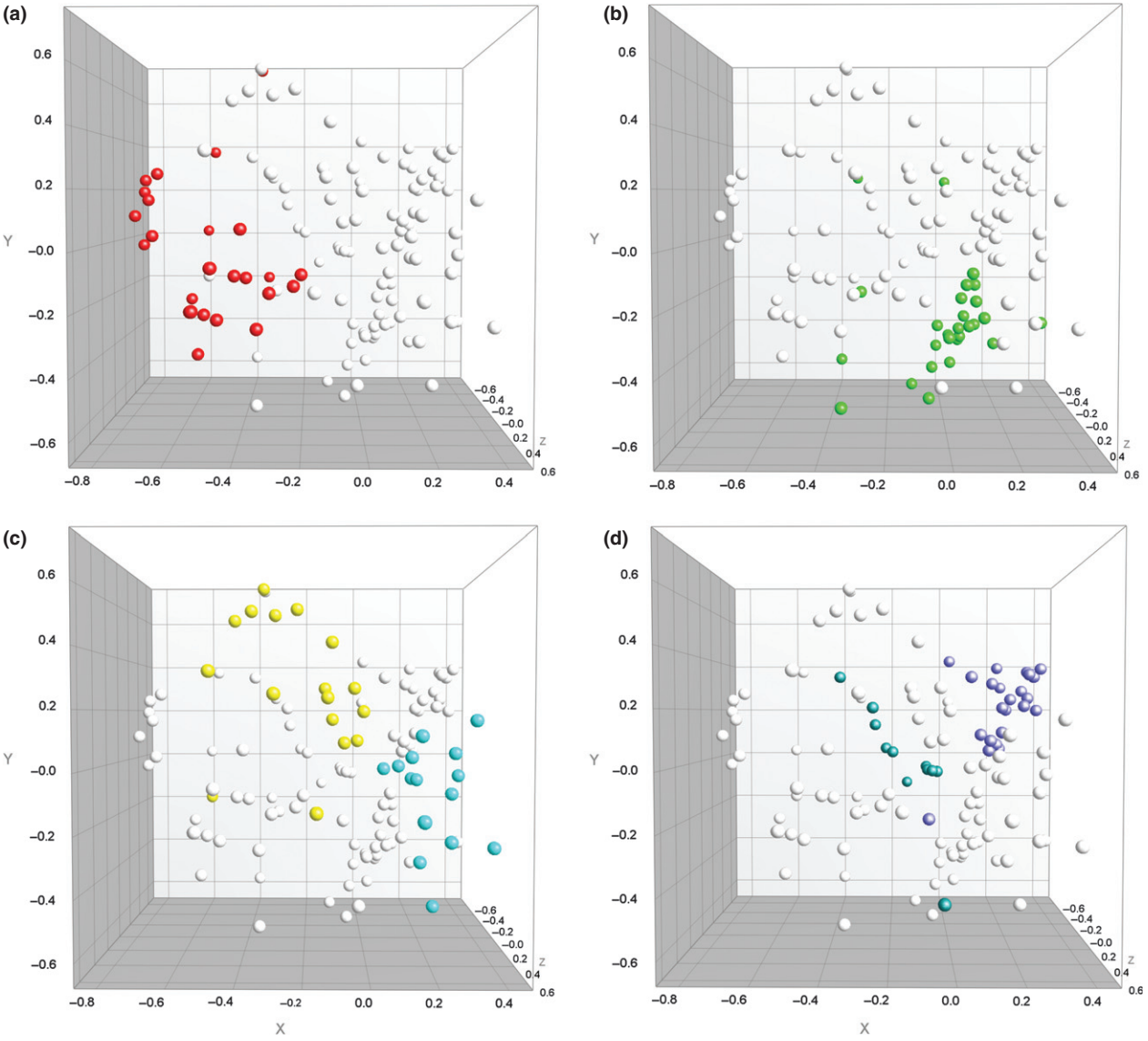

Fig. 3. MDS representation of the similarity matrix generated by RISA cluster analysis. Each of the skin microbiota is represented by a dot, and the distance between dots represents relatedness obtained from the similarity matrix. Isolates are colored based on fish species. In (a), only the microbiota from red snapper (*Lutjanus campechanus*) are highlighted. (b) Displays the microbiota from Atlantic croaker (*Micropogonias undulatus*). (c) Shows the microbiota from striped mullet (*Mugil cephalus*; yellow) and pinfish (*Lagodon rhomboides*; turquoise). (d) Highlights the microbiota from spotted seatrout (*Cynoscion nebulosus*; teal) and sand seatrout (*Cynoscion arenarius*; purple).

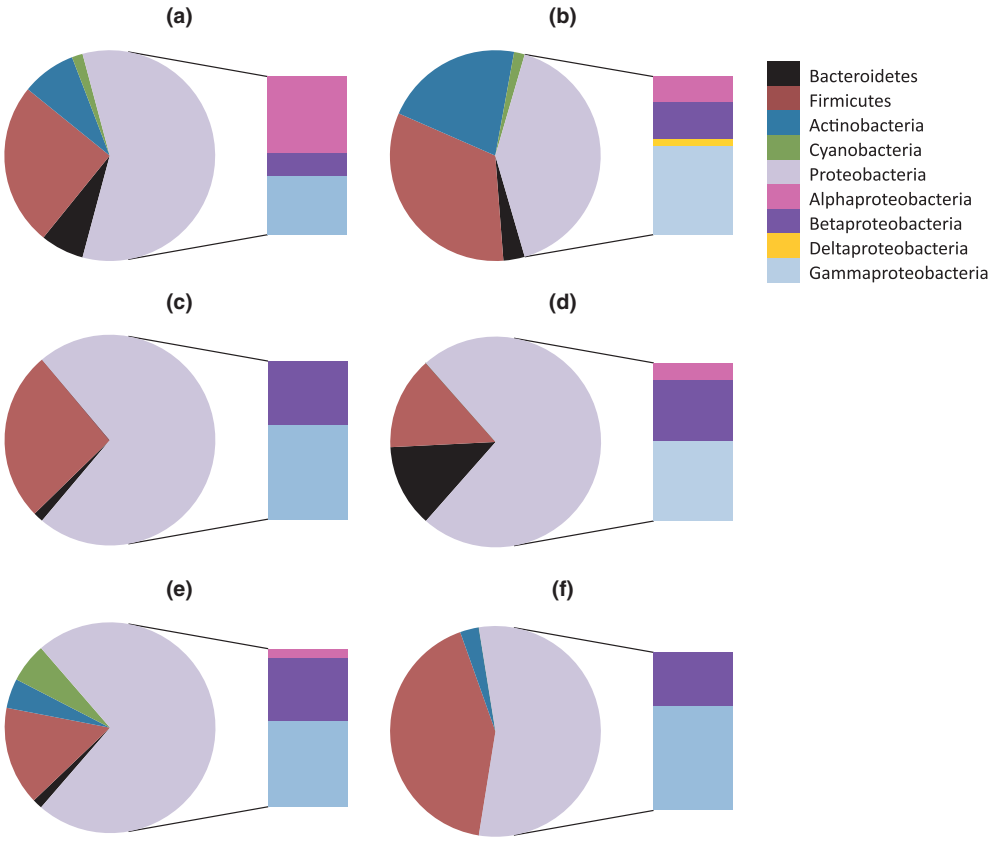

Fig. 4. Bacterial diversity at the phylum level (pie chart) and class level (bars) based on 16S rRNA gene sequencing. Pie diagrams show percent of sequenced clones belonging to different bacterial phylum from each fish species analyzed. Bar graphs represent the percentage of *Proteobacteria* classes detected in each fish species. (a) Striped mullet (*Mugil cephalus*); (b) red snapper (*Lutjanus campechanus*); (c) spotted seatrout (*Cynoscion nebulosus*); (d) sand seatrout (*Cynoscion arenarius*); (e) pinfish (*Lagodon rhomboides*); (f) Atlantic croaker (*Micropogonias undulatus*).

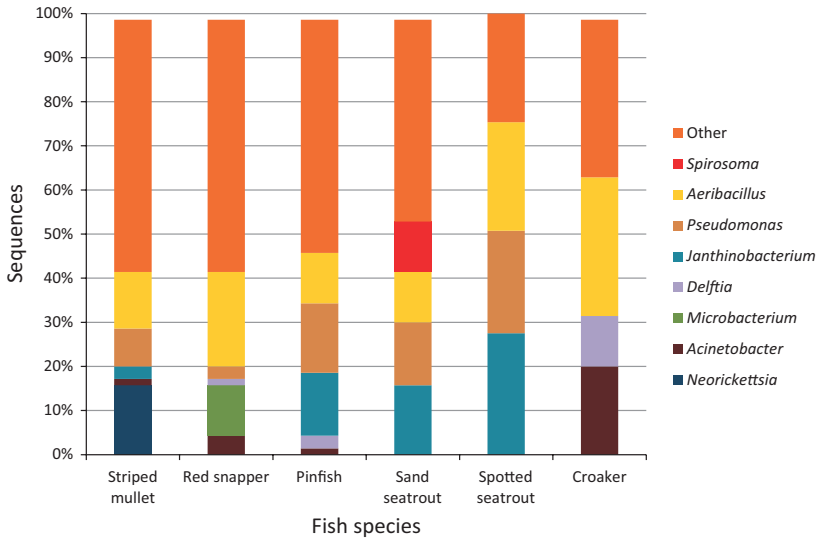

Fig. 5. Distribution of predominant bacterial genera in each fish species based on 16S rRNA gene sequencing.

snapper and Atlantic croaker. Unique bacterial genera associated with specific fish species included *Neorickettsia* on striped mullet and *Microbacterium* in red snapper. Spotted seatrout showed the least diverse bacterial population, with > 75% of all the sequences recovered from this species belonging to three genera. Red snapper and striped mullet displayed the most diverse microbiota (at genus level), having representatives of 12 genera each.

Discussion

Oceans are oligotrophic environments wherein nutrients are scarce for heterotrophic bacteria. From that perspective, fishes are nutrient islands in a vast, predominantly nutrient-poor sea. From fish eggs to adults, fish surfaces are immersed in water and thereby susceptible to colonization by aquatic bacteria. This process appears to be selective because specific microbiota have been associated with wild fish larvae (Jensen *et al.*, 2004) as well as with hatchery-reared fish (Olafsen, 2001). Based on laboratory experiments, adhesion to fish skin appears to be a widespread trait among bacteria, although these studies focused on fish pathogens like species of *Vibrio* (Larsen *et al.*, 2001) and *Flavobacterium* (Olivares-Fuster *et al.*, 2011). In addition, some bacteria are positively chemotactic to fish mucus (Larsen *et al.*, 2001; Klesius *et al.*, 2008). Because fish mucus is nutrient-rich (Shephard, 1994), and bacteria are capable of growing in it (Garcia *et al.*, 1997), marine bacteria may benefit from attaching to fish skin, which is a surface that is normally covered by a contiguous layer of mucus (to the extent that some anatomic treatments of fish skin refer to the mucus layer as a 'cuticle' on the same functional anatomic footing as the keratinized epidermis of terrestrial vertebrates; Ferguson, 2006). However, from the host's perspective, bacterial adhesion to skin should be mediated to avoid over-colonization and disruption of integument functions. This is accomplished, probably in part, by the constant sloughing of the upper layers of the epidermis and the continuous secretion of mucus.

The equilibrium between bacteria that adhere to skin and the number of bacteria that an apparently healthy host can support will play a role in determining the 'normal skin microbiota' for a particular fish species. The diversity and structure of those microbiota can be studied at three levels: alpha diversity (within a host), gamma diversity (within a population), and beta diversity (that observed between hosts of the same population; Robinson *et al.*, 2010). The use of RISA, a rapid and inexpensive method, allowed us to compare the microbiota from each individual fish without the need for pooling samples and thus missing the host-to-host (beta) diversity. Although RISA does not provide phylogenetic information on particular amplified sequences, the complexity of RISA profiles reflects that of the microbiota (Fisher & Triplett, 1999). Our RISA results revealed a broad range of similarities within all the samples analyzed at both intra- and interspecies levels (Fig. 2). Not all microbiota from the same fish species clustered together; therefore, we observed nonzero beta diversity among the populations examined. Based on our previous experience with RISA (Arias *et al.*, 2006; Sudini *et al.*, 2011), we concluded that the observed diversity was not due to the variability introduced by the technique with the set cutoff point for describing separate clusters at 70% similarity. Nonzero beta diversity can result from random or nonrandom colonization patterns; however, there is increasing evidence in support of the latter (Robinson *et al.*, 2010). In terms of relating the observed beta diversity with the variables examined, the defined clusters could not be assigned to a specific date or location. However, when all pairwise similarities within a species were compared by ANOSIM, both variables (location and date) significantly influenced the microbiota profiles. Although our data does not refute the previously proposed hypothesis by which bacterial communities on fish are a result of the bacteria present in their surrounding waters (Nguyen *et al.*, 2008; Wilson *et al.*, 2008; Smith *et al.*, 2009), it suggests that fish species has greater influence on external microbiota.

The structure of marine bacterial communities is a result of both habitat (spatial) filtering (Pontarp *et al.*, 2012) and temporal patterns influenced by both biotic and abiotic factors (Fuhrman *et al.*, 2006). With exception of red snapper, an obligate marine species typically associated with offshore reefs (Topping & Szedlmayer, 2011), all fishes analyzed in this study comprise common residents of estuarine waters (Carpenter, 2001). We expected that geographic location would not significantly determine the studied microbiota to the extent that season would (throughout the study water temperature fluctuated between 16 and 32 °C). However, both variables exerted a similar influence on skin microbiota based on the global *R*-values obtained. Interestingly, red snapper microbiota were divided into two clusters: One cluster was the most basal group in the RISA dendrogram, and the other clustered with a pinfish sample collected from Orange Beach 10 months earlier. Both fish species were collected from distinct environments (offshore vs. coast) yet their bacterial profiles shared up to 30% similarity. Nevertheless, red snapper microbiota were the least similar to all other fish species, which may be explained by the different habitats in where those fish were collected (offshore vs. coast).

The variable 'fish species' had a global *R*-value of 0.549, and therefore, most significantly shaped the structure of the fish microbiota. This result did not refute the notions that (1) the host plays an active role in selecting

which microbial taxa can colonize and persist on it or that (2) the constituents of the microbiota are highly specific to particular host fish, similar to microbial species that only will grow on a particular kind of culture medium. A long list of physiologic attributes of the bacterium or the fish could explain this specificity, and we did not specifically test any of them. We speculate that differences in mucus composition (Shephard, 1994) and antimicrobial properties (Subramaniam *et al.*, 2008) between fish species may mediate adhesion interactions between fish and bacteria. Clearly, and contrary to our initial hypothesis, the variable 'fish species' determines the structure of the fish skin microbiota more so than the abiotic factors temperature or salinity; both of which reportedly are predictive of marine bacterioplankton microbiota (Fuhrman *et al.*, 2006; Pontarp *et al.*, 2012).

As RISA does not provide phylogenetic information on the microbiota composition, sequencing was conducted to obtain information on the predominant bacteria associated with skin and mucus of the six species examined. Sequence data showed that each fish species had a unique microbiota. Overall, the *Proteobacteria* was the predominant phylum colonizing the external surface of fishes with 61% of all sequences belonging to this phylum. This result is in agreement with previous studies on other species regardless of the technique used for bacterial identification (Colwell & Liston, 1962; Horsley, 1977; Wilson *et al.*, 2008). Within the phylum *Proteobacteria*, the *Gammaproteobacteria* was the most abundant class in all fish species except the striped mullet, and *Aeribacillus* was the most frequently identified genus. *Pseudomonas* was also frequently identified, and it is noteworthy that previous studies using either culture or culture-independent methods have also identified members of *Pseudomonas* as the main component of the skin microbiota of cod (*Gadus* spp.; Georgala, 1953; Wilson *et al.*, 2008), salmon (*Salmo salar*; Horsley, 1973), skate (*Raja* spp.), lemon sole (*Microstomus* spp.), herring (*Clupea* spp.; Horsley, 1977), surgeon fish (*Acanthurus triostegus*), jack (*Caranx ferdau*), and grouper (*Epinephelus merra*) from the Pacific Ocean (Colwell & Liston, 1962). Other frequently isolated genera such as *Janthinobacterium* and *Acinetobacter* have been previously reported from fish (Jensen *et al.*, 2004; Austin, 2006).

Aeribacillus (ph. *Firmicutes*) was identified in all fish species we surveyed, comprising the first report of it in association with a fish. The sequence identities obtained after BLAST identified the majority of our *Aeribacillus* sequences as *Aeribacillus pallidus* (percent identity match at 98% or higher to type strain DSM 3670). This was a surprising result because this species is known to be a thermophilic, halotolerant bacteria found in hot springs. We queried the GenBank database with 16S rRNA gene sequences that were 400 bp in length or longer, and the BLAST results were unequivocal. It is possible that our sequences may represent a new species of *Aeribacillus*, the full 16S rRNA gene sequence will be required to support this, or that we have discovered a new ecological niche for *A. pallidus*.

Noteworthy also was the presence of *Neorickettsia* sp. in striped mullet, an intracellular pathogen that causes severe illnesses in mammals and that is transmitted by flukes (*Platyhelminthes*: *Digenea*) that infect fishes (Vaughan *et al.*, 2012). The sequence identity was 95–96% with those found in GenBank (closest match was *Neorickettsia risticii* type strain ACTT VR-986 in all cases), which suggested a potential new species of *Neorickettsia* associated with striped mullet. Similarly, *Microbacterium* sp. was found in red snapper only, yet represented up to 11% of all bacterial sequences from all red snappers sampled. Sequence identities were high in most cases with percent identities over 98% matching *Microbacterium arborescens* (type strain DSM 20754), *Microbacterium esteraromaticum* (type strain DSM 8609), and *Microbacterium paraoxydans* (type strain DSM 15019). However, five sequences shared < 97% sequence identity with GenBank entries and may represent new *Microbacterium* species.

Predominant marine bacteria genera such as *Vibrio* and *Photobacterium* were identified in extremely low frequency (*Photobacterium*) or not detected at all (*Vibrio*). This contradicts previous studies in which both genera were abundant and common (Jensen *et al.*, 2004; Smith *et al.*, 2007; Wilson *et al.*, 2008). Interestingly, these studies utilized fingerprint techniques followed by 16S rRNA gene sequencing, similar to our methods. However, in those studies, fish were collected by trawling, which increases bacterial densities on skin (Austin, 2006). Differences in fishing gear may influence the recovery of those bacteria loosely associated with skin and mucus.

In conclusion, this study provides evidence for the presence of specific external microbiota associated with particular fish species. The composition and structure of those microbiota are likely to be impacted by several cofounding variables including abiotic factors linked to geographic locality and season as well as biotic factors related to the nutrient potential or antimicrobial components of fish mucus. The bacterial profiles obtained from individual fish showed nonzero beta diversity, indicating that the host influences the bacterial taxa associated with its external surfaces. In addition, and based on our sequence data, we suggest that the external surfaces of fish are colonized by a microbiota that is distinguishable from fish to fish species.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. RISA profiles obtained from seawater samples indicating location and collection date.