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REPORT

Invasive lionfish harbor a different external bacterial community than native Bahamian fishes

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Abstract The introduction and subsequent spread of lionfish into the Atlantic Ocean and Caribbean Sea has become a worldwide conservation issue. These highly successful invaders may also be capable of introducing non-native microorganisms to the invaded regions. This study compared the bacterial communities associated with lionfish external tissue to those of native Bahamian fishes and ambient water. Terminal restriction fragment length polymorphism analyses demonstrated that lionfish bacterial communities were significantly different than those associated with three native Bahamian fishes. Additionally, all fishes harbored distinct bacterial communities from the ambient bacterioplankton. Analysis of bacterial clone libraries from invasive lionfish and native squirrelfish indicated that lionfish communities were more diverse than those associated with squirrelfish, yet did not contain known fish pathogens. Using microscopy and molecular genetic approaches, lionfish eggs were examined for the presence of bacteria to evaluate the capacity for vertical transmission. Eggs removed from the ovaries of gravid females were free of bacteria, suggesting that lionfish likely acquire bacteria from the environment. This study was the first examination of bacterial communities associated with the invasive lionfish and indicated that they support different communities of environmentally derived bacteria than Caribbean reef fishes.

Keywords Lionfish · Fin-associated bacteria · Invasive · T-RFLP · Clone library

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Introduction

Since the mid-1980s, the US Atlantic coast and the Caribbean Sea have experienced rapid population growth and dispersal of the invasive lionfish (*Pterois volitans/miles* complex), resulting in a current population density that is estimated to be five times greater than that found in portions of its native range in the Indo-Pacific (Green and Côté 2009). Mitochondrial analysis of specimens from a region of the invaded range indicated that 8–12 individuals likely gave rise to the invasive population (Betancur et al. 2011), creating a strong founder effect that limited the genetic variability within lionfish individuals (Hamner et al. 2007; Freshwater et al. 2009). Multiple aquarium releases or escapes along the Florida coast are thought to have been the source of the founding individuals (Morris and Akins 2009), and populations have now been established as far north as Cape Hatteras, NC (USA), south to the coast of Venezuela, west into the Gulf of Mexico, and east to Bermuda (Schofield 2009).

The ecological impact of lionfish has been significant throughout the invaded region. Their aggressive feeding lifestyle coupled with a non-selective diet is thought to have contributed to an approximately 80 % decrease in native fish recruitment on artificial reefs when lionfish are present (Albins and Hixon 2008; Côté and Maljković 2010). Lionfish utilize a range of habitats including mangroves, sand flats, and deep water reef walls (Barbour et al.

2010; Lesser and Slattery 2011; Kulbicki et al. 2012), in addition to their use of reef cracks and crevices. The expansion of lionfish throughout the Atlantic Ocean is the first incidence of a non-native marine fish threatening the coral reef ecosystem (Albins and Hixon 2011), and an effective control mechanism has yet to be proposed.

In his book, Diversity of Life, E.O. Wilson listed the "four mindless horsemen of the environmental apocalypse," which include the introduction of non-native species and the spread of disease agents by these introduced species (Wilson 1992). In the past, invasive species have introduced non-native microorganisms into the new systems, exacerbating their already deleterious effects on the ecosystem (Tompkins et al. 2003; Crowl et al. 2008). Both the documented ecological effects and the potential for introduction of microorganisms imposed by the lionfish invasion have resulted in their presence being recognized as one of the world's most pressing conservation issues (Sutherland et al. 2011).

While the extent of the impact of the lionfish invasion into the Atlantic Ocean and Caribbean Sea is beginning to be realized, knowledge of the microbial communities associated with the species complex is sparse. To fully understand the potential for an invasive species to spread disease throughout the invaded range by introduction of non-native microorganisms, potential agents of disease, examinations of the holobiont (i.e., the entire community of living organisms residing in or on a macroorganism) must be first conducted (Crowl et al. 2008). Fish harbor bacterial communities on their external surfaces that have been shown to function in disease resistance (Chabrillón et al. 2005) and drag reduction (Bernadsky and Rosenberg 1992). Despite this, studies comparing bacterial communities between fish species remain limited to early conclusions drawn from culture-dependent methods (Colwell 1962; Liston and Colwell 1963; Cahill 1990). Thus, the foci of this study were to characterize the bacterial communities associated with external surfaces of lionfish and compare the resident communities to those found associated with three species of native Bahamian fish (squirrelfish *Holocentrus adscensionis*, white grunt *Haemulon plumierii*, and lane snapper *Lutjanus synagris*) as well as in the ambient water column. The native fishes used in this study are reef-associated species that feed on smaller fishes and crustaceans, thereby occupying the same general habitats and trophic position as the invasive lionfish.

Fish acquire their associated microbial communities through several possible mechanisms: vertically via transmission from their parent organisms, horizontally from exposure to microorganisms within the ambient environment, or through a combination of both vertical and horizontal transmission (Hansen and Olafsen 1989). If lionfish acquire their associated microbial communities vertically, it suggests a greater possibility for introducing novel organisms, including potential pathogens, to the invaded range. To address these questions, a combination of terminal restriction fragment length polymorphism analyses and clone library construction was employed to provide resolution of the associated bacterial communities. In order to evaluate the acquisition of microorganisms, lionfish eggs removed from gravid females were analyzed for the presence of bacteria using molecular genetic techniques and electron microscopy.

Materials and methods

Specimen collection

Healthy fish specimens and ambient water were collected by scuba diving from patch reefs surrounding Lee Stocking Island, Bahamas, in conjunction with studies examining eukaryotic parasitism (P. Sikkel, pers. comm.). Fin clips from net caught lionfish ($n = 15$) and native fishes ($n = 6$) were taken from pectoral fins, preserved in 1.8 ml RNAlater (Ambion), and stored at −20 °C until laboratory analysis. Five 500 ml seawater samples were filtered through 0.2 μm polycarbonate Millepore filters, and the filters were preserved in 1.8 ml RNAlater and stored at −20 °C. To sample lionfish eggs, ovaries were extracted from pithed gravid female lionfish ($n = 2$) within 2 h, eggs were removed and preserved in 1.8 ml RNAlater prior to being stored at −20 °C.

DNA extraction and amplification

Total community DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's protocol with one exception. Cell lysis was performed using an overnight incubation at 37 °C rather than the recommended 3 to 4 h at 56 °C. Universal prokaryotic primers 8F (5′-AGAGTTTGATCMTGGCTCAG-3′; Edwards et al. 1989) with and without a fluorescent *S*-hexachlorofluorescein (HEX) label and 1392R (5′-ACGGGCGGTGTGTACA-3′; Lane 1991) were used to amplify an approximately 1,385-bp region of the 16S rRNA gene by polymerase chain reaction (PCR). Each reaction consisted of 2 U Omni Klen*Taq* (Klentaq), 1× PCR buffer, 1.25 mM $Mg(OAc)_2$, 0.06 mM deoxynucleoside triphosphates, 0.8 μg bovine serum albumin (BSA), 25 pmol of each primer, and sterile deionized water to a final volume of 100 μl. Reaction conditions were 85 °C for 5 min, followed by 30 cycles of 94 °C for 45 s, 62 °C for 90 s, and 72 °C for 90 s, with a final 10-min extension at 72 °C. Negative controls containing all reagents but no sample DNA were run with each reaction. Amplification products were subjected to electrophoresis on 1 % agarose gels containing GelRed™ (Biotium)

for 70 min at 70 mV and visualized under UV transillumination with a gel imaging system (Fotodyne).

Terminal restriction fragment length polymorphism analysis

Each sample was amplified in triplicate using the HEX label on the 5′ end of the 8F primer for downstream terminal restriction fragment length polymorphism (T-RFLP) analysis. The 3 products per sample were cleaned individually with a QIAquick PCR clean-up kit (Qiagen) and the eluates pooled. Digestion reactions were carried out using 400 ng of cleaned PCR product, 2 U of the restriction endonuclease *Hae*III (New England BioLabs), 1 × enzyme buffer, and sterile deionized water to a total volume of 50 µl. Reaction mixtures were incubated for 8 h at 37 °C followed by a 30-min enzyme deactivation at 80 °C before being stored at 4 °C. Digestion products were ethanol-precipitated overnight and centrifuged, and the pellet dried in a Jouan RC1022 centrivac (Thermo Scientific). Pellets were resuspended in 10 µl of deionized formamide and 0.5 µl of 6-carboxytetramethylrhodamine size standard (Applied Biosystems) prior to being analyzed with an ABI 310 Genetic Analyzer with a 50-cm capillary array (Applied Biosystems). Terminal restriction fragment (T-RF) lengths were determined using the Local Southern size-calling algorithm of the GeneScan v3.1 analysis software (Applied Biosystems).

Data matrices were constructed using peaks above a threshold of 50 fluorescence units, which was considered to be the background level. Peaks smaller than 100 bp and >500 bp were removed from the data set to avoid uncertainties associated with fragment size determination. To determine which peaks were further analyzed, the variable threshold method (Osborne et al. 2006) was employed. Using TREX (Culman et al. 2009), the resulting profiles were binned at a 0.5 clustering threshold prior to statistical analysis.

Clone library production

DNA extracted from 3 lionfish and 3 squirrelfish samples was PCR amplified without the HEX label on the forward primer. To reduce the presence of chimeric sequences, a reconditioning PCR was performed using 2 µl of the original PCR product in a fresh reaction mixture amplified for an additional three cycles at the same times and temperatures (Thompson et al. 2002). Following PCR, electrophoresis, and visualization of the samples, bands of the appropriate size were excised with sterile razor blades and cleaned with a Gel Extraction clean-up kit (Qiagen) according to the manufacturer's protocol. Using the TA Cloning Kit (Life Technologies), 2 µl of the PCR products was ligated into vector pCR®2.1 and transformed into chemically competent *Escherichia coli* cells following the manufacturer's instructions.

Transformed colonies were screened by restriction enzyme analysis. Briefly, DNA was extracted from each colony via bead beating in a 5 % Chelex 100 resin (Bio-Rad Laboratories) in sterile water solution, and the 16S rRNA genes amplified by PCR as described above but without BSA in the reaction mixture; 20 µl of PCR products was digested with *Hae*III for 8 h at 37 °C followed by a 30-min incubation at 80 °C. Digested DNA was subjected to electrophoresis on 1 % agarose gels containing GelRed™ with a molecular weight ladder. The TotalLab TL100 1D v2009 computer software package (Nonlinear Dynamics Ltd.) coupled with visual analysis was used to group clones into operational taxonomic units (OTUs) based on the sizes of resulting bands, and a representative clone from each OTU was selected. Plasmids ($n = 186$) were purified from selected clones using the E.Z.N.A Mini-prep kit (Omega Bio-Tek) and bi-directionally sequenced by Functional Biosciences. Sequences were aligned using BioEdit v. 7.0.9.0 (Hall 1999) and compared to the NCBI database using BLASTn searches (Altschul et al. 1990). Potential chimeric sequences were removed using the UCHIME algorithm (Edgar et al. 2011), as implemented in MOTHUR (Schloss et al. 2009). The Silva.gold alignment was used as a reference data set for chimera analysis. Sequences were submitted to GenBank under the accession numbers JX680687–JX680801.

Resulting sequences were in silico digested with *Hae*III, and the length of the terminal 8F fragment was calculated. Based on previous work demonstrating the impact of the HEX label on migration following digestion with *Hae*III, 2 bp was added to predicted T-RFs. The predicted and observed T-RFs were then compared to match individual T-RFs with specific clone sequences.

Statistical analysis

Bray–Curtis similarity matrices were constructed using fourth root transformations of the normalized T-RFLP peak area data and analyzed with PRIMER v.6 software (Clarke and Gorley 2006). Paired *t* tests were used for comparisons of bacterial communities between species. A one-way analysis of similarity (ANOSIM) was used to examine the effect of fish type on bacterial community composition and to determine whether bacterial communities associated with fish were different from bacteria present in the ambient water. Non-metric, multidimensional scaling (MDS) plots allowed for visualization of bacterial community composition between water, native fish, and lionfish samples in two-dimensional space. One-way similarity

percentages (SIMPER) were used to calculate the average contribution of each T-RF to the total community composition within and between treatment groups.

Rarefaction curves were generated with MOTHUR v 1.26 (Schloss et al. 2009) using the OTUs determined from the restriction enzyme digestion patterns of the clones. All clones that yielded a particular pattern were included with the sequenced clone from that OTU in the analyses; thus, substantially more clones than were sequenced were included in the rarefaction and diversity analyses. Rarefaction curves were created for lionfish and squirrelfish OTUs using species definitions of 99, 97, and 95 % sequence identity. The Chao1, Shannon, and Simpson diversity indices were calculated within MOTHUR at the same species definitions. LibShuff statistical analysis was used within MOTHUR (Schloss et al. 2009) to test for statistical differences between bacterial community compositions of the two fish types. This is a generic test using the Cramer-von Mises test statistic with the default 10,000 random permutations (Schloss et al. 2009).

Molecular genetic and microscopic analyses of lionfish eggs

Using a dissecting microscope (Tritech Research, Inc.), RNAlater-preserved eggs ($n = 5$) were removed from the surrounding matrix and ~5 eggs were maintained within the matrix for each fish. Total community DNA was extracted from each subsample using the DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's instructions. Universal prokaryotic primers 8F and 1392R were used for PCR amplifications as described previously. DNA extracted from adult lionfish fins and pure cultures of marine bacteria was amplified concurrently as positive controls.

To inspect the external egg surface and internal ovarian matrix for the presence of bacterial cells, eggs ($n = 3$) and eggs within the ovary matrix ($n = 3$) were fixed in a 2.5 % glutaraldehyde in 0.1 M sodium phosphate buffer solution (pH 7.4) for electron microscopy. Secondary fixation was conducted using 2 % osmium tetroxide in sodium phosphate buffer. For scanning electron microscopy, samples were dehydrated in ethanol, critical point dried, and coated with gold palladium. Eggs were examined using a Hitachi S-2500 scanning electron microscope. For transmission electron microscopy, fixed samples were dehydrated in ethanol. Spur's solution was used to infiltrate the samples, which were then embedded and polymerized. Embedded eggs were sectioned using a Leica EM UC6 Ultramicrotome and stained using uranyl acetate. Sections were viewed on a Hitachi H-7650 transmission electron microscope.

Results

T-RFLP

T-RFLP analysis was used to assess the relative diversity and richness of the bacterial communities associated with the external surfaces of lionfish and native fishes and within the ambient water column. Because T-RFs can represent multiple species with a shared restriction endonuclease digestion site, individual peaks should be considered OTUs rather than species. The bacterial communities associated with lionfish yielded significantly more T-RFs (mean number of T-RFs = 85.9; t test $p < 0.05$) than native fishes (64.9 T-RFs), suggesting that lionfish support a more diverse bacterial community than native fishes.

ANOSIM showed no significant difference between the bacterial communities associated with the 3 native fish species (data not shown). Additionally, pairwise comparisons between the bacterial communities of native fishes showed no significant differences ($p > 0.05$), supporting the grouping of native fishes for ANOSIM analyses. One-way ANOSIM indicated that the bacterial communities associated with lionfish were significantly different than those associated with native Bahamian fishes ($R = 0.47$, $p < 0.001$). Both lionfish ($R = 0.58$, $p < 0.001$) and native fishes ($R = 0.72$, $p < 0.001$) harbored significantly different bacterial communities than those found in the ambient seawater.

Ordination plots show fish-associated (lionfish and native fishes) bacterial communities clustered separately from those in the water column, suggesting that fish fin surfaces support a distinct bacterial community from the bacteria found in their aquatic environment (Fig. 1). Although several lionfish samples are found between the samples of the native fishes, there is reasonable separation of the two fish groups. The relatively high stress value of the MDS plot (>0.2) typically indicates that a higher dimensionality plot should be used, but the lack of scatter around the regression line of the Shepard diagram (not shown) and the statistical support provided by the ANOSIM analyses suggest that the placement of samples in two-dimensional space may still provide a useful visualization of bacterial community composition.

To determine the role of individual T-RFs in contributing to the separation between groups of samples, SIMPER was employed. In all cases, differences between the bacterial communities were not driven by a single T-RF, as the top contributors to the dissimilarity between groups accounted for <6 % of the total, suggesting that multiple taxa differ among groups. Lionfish-associated bacterial communities were 81.3 % dissimilar to those associated with native fishes, while the fish-associated bacterial communities were each highly dissimilar to ambient

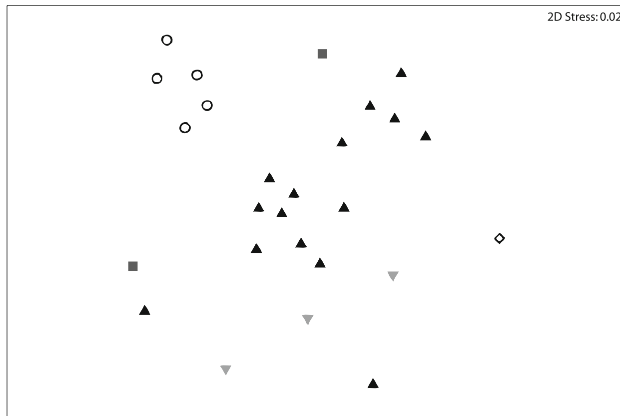

Fig. 1 MDS plot showing the distribution of the bacterial communities associated with invasive lionfish (*black triangles*), native squirrelfish (*light gray inverted triangles*), white grunt (*black squares*), Lane snapper (*open diamond*), and ambient water (*open circles*) in two-dimensional space

bacterioplankton (81.6 and 85.9 % dissimilarity for lionfish and native fish communities, respectively).

Clone libraries

Clone libraries generated from lionfish and squirrelfish fin samples revealed that both types of fishes supported diverse bacterial communities (Electronic Supplemental Material, ESM Table 1) and that community structure significantly differed (LibShuff, $p < 0.0001$) between the fishes. Within the native fishes, squirrelfish were selected for comparison because they occupy the most similar habitat to the lionfish. A total of 514 clones (297 from squirrelfish and 217 from lionfish) were evaluated using restriction endonuclease digestions to identify unique banding patterns to maximize the information recovered from sequencing efforts. From these clones, 84 and 86 were selected for sequencing from lionfish and squirrelfish samples, respectively. After removal of chimeric sequences, sequences were compared to the GenBank database using BLASTn searches to identify the closest matches. *In silico* digests with the restriction endonuclease *Hae*III on the sequences demonstrated that all clones with a terminal fragment within the detectable range (100–500 bp) were recovered in our T-RFLP analyses (ESM Table 1). The majority of the clones were most similar to members of the Proteobacteria, with representatives from γ-Proteobacteria (53.9 %), α-Proteobacteria (14.2 %), and ß-Proteobacteria (9.7 %). Clones related to the Bacteroidetes (14.2 %), Firmicutes (2.6 %), and Fusobacteria (2.6 %) were also recovered.

A number of clones from both libraries were most closely related to species within the phylum Bacteroidetes, family Rhodobacteraceae, and genera *Alteromonas*, *Cobetia*, *Marinomonas*, *Nonlabens*, *Pseudoalteromonas*, *Sandarakinotalea* (now unified into the genus *Nonlabens*; Yi and Chun 2012), *Serratia*, *Tenacibaculum*, and *Vibrio*. Among the genera that differed between the libraries, multiple squirrelfish clones were most closely related to the known fish pathogens *Photobacterium damselae* (de La Banda et al. 2010) and *Clostridium* spp. (Austin and Austin 1999). No common fish pathogens were recovered from lionfish tissue. Clones most closely related to *Serratia marcescens*, a known coral pathogen (Sutherland et al. 2011), were common in the squirrelfish library, accounting for 22 % of the analyzed clones (54 of 238 clones). This species was also reported in the lionfish clone library, but at a much lower incidence relative to squirrelfish (14.9 %, 18 of 121 clones).

A suite of indices was used to analyze the relative diversity and richness of bacterial communities associated with lionfish and squirrelfish (Table 1) using all screened clones. These results support the species assemblage curves (Fig. 2), which also indicated that additional bacterial OTUs were present but not sampled in both libraries. Shannon and Simpson indices estimated the richness and diversity of the bacterial communities that were sampled (Table 1). Using the Shannon index, both lionfish- and native fish-associated bacterial communities showed high richness and evenness (2.84 and 2.13, respectively, using a species definition of 97 % sequence similarity). Bacterial diversity was also high in both lionfish and squirrelfish-associated samples as reported by the Simpson index (0.07 and 0.22, respectively, for a 97 % species definition), indicating that two randomly selected bacteria had a very low likelihood of being the same species. While bacterial communities from both fish types were diverse, the Shannon and Simpson indices suggested that lionfish supported a slightly more diverse bacterial community than squirrelfish.

Microscopic and molecular genetic analysis of lionfish eggs

The prokaryotic 16S rRNA gene was not amplified by PCR from lionfish eggs or from lionfish eggs within ovary matrix despite being successfully amplified from adult lionfish fin tissue (data not shown). Further, bacteria were not visualized under scanning (Fig. 3) or transmission electron microscopy (data not shown) of lionfish eggs.

Discussion

During the mid-1900s, a number of studies were conducted on the normal bacterial flora of marine fish found in the North Sea (Liston 1956, 1957; Georgala 1958), north Atlantic (Dyer 1947), north Pacific (Snow and Beard 1939;

Table 1 Diversity indices for lionfish and squirrelfish bacterial communities using 99, 97, and 95 % sequence similarity for operational taxonomic unit definitions

Sample	# of OTUs	Chao1 index	Recovered (%)	Shannon index	Simpson index
Lionfish					
99 %	32	48.5	66.0	3.11	0.05
97 %	26	33.2	78.3	2.84	0.07
95 %	21	24.8	84.8	2.68	0.08
Squirrelfish					
99 %	31	57.3	54.1	2.22	0.22
97 %	26	59.0	44.1	2.13	0.22
95 %	25	52.5	47.6	2.12	0.22

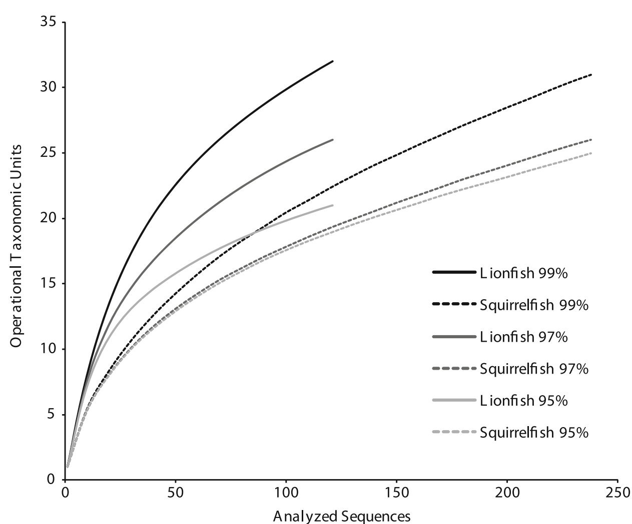

Fig. 2 Species accumulation curves based on the number of clones within each operational taxonomic unit (OTU). Species definitions for an OTU were set at 99, 97, and 95 % sequence similarity. The values for the *X axis* reflect the number of clones screened by restriction endonuclease digestion to identify novel banding patterns (i.e., OTUs) rather than the number of clones sequenced

Kiser and Beckwith 1944), and the central Pacific (Colwell and Liston 1962) using cultivation methods. These studies isolated bacteria that were identified mostly to the genus level using biochemical assays. Regardless of the ocean where fish were collected, results from cultivation-based studies of the microflora on fish skin indicated that members of the genera *Pseudomonas*, *Achromobacter*, *Moraxella*, *Acinetobacter*, and *Vibrio* were common constituents (Colwell 1962; Horsley 1973; Gilmour et al. 1976; Austin 1983). The frequency of isolation of the various genera of bacteria from different fish species varied between sampling locations but these studies, with the exception of Austin (1983), indicated that the major components of the skin microflora reflected what was present in the ambient water column.

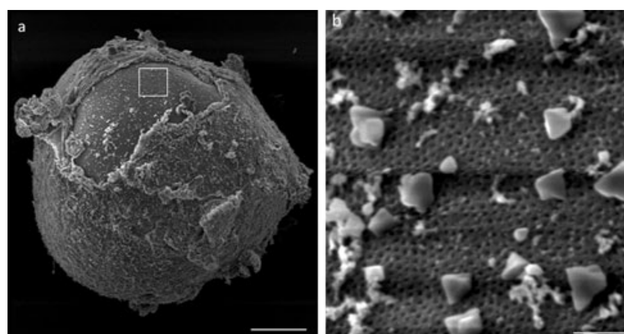

Fig. 3 Scanning electron micrographs of a lionfish egg. **a** Lionfish egg with mucus layer pulled back; *scale bar* equals 69 µm. **b** Egg surface in area indicated by *white box* in **a** showing the lack of bacterial cells on the egg surface; *scale bar* equals 2.55 µm

The majority of the more recent work regarding fish-associated microbial communities has been performed on reared fishes, with particular emphasis on characterization of the intestinal flora and the application of probiotics (e.g., Bergh 1999; Griffiths et al. 2001; Jensen et al. 2004). Few investigations have been conducted on the skin microflora of wild fish (Colwell 1962; Horsley 1973; Bernadsky and Rosenberg 1992), and these were conducted using culture-dependent methods. This study examined differences between the bacterial communities associated with lionfish and native Caribbean fishes using molecular fingerprinting techniques as a first step in evaluating the lionfish holobiont as a potential vector for the introduction of non-native bacteria.

Using T-RFLP and clone library analyses, diverse but different communities of bacteria were found on the fins of invasive lionfish and 3 native Caribbean fishes. These results support the conclusions of Cahill (1990), who also noted that the microflora of fish skin varies in marine species. Our results demonstrate that although the 3 Caribbean fishes shared similar external bacterial communities, there were distinct differences between those communities and lionfish-associated bacteria and ambient bacterioplankton. All samples were collected at the same time from the same patch reef environments, suggesting that fish species were selectively colonized by bacteria as each was exposed to the same pool of bacteria in the surrounding water. These findings contradict what was reported for culture-based studies that found no host specificity within skin bacteria (Colwell 1962; Liston and Colwell 1963), and reports suggesting the major components of the skin flora were similar to those present in the ambient water (e.g., Horsley 1973; Gilmour et al. 1976). These early cultivation-based studies likely underestimated the bacterial community diversity that is captured with culture-independent molecular methods; however, additional culture-independent studies from other regions are

needed to confirm the specificity of bacterial communities associated with different fishes.

Contrary to early cultivation studies where *Pseudomonas* spp. dominated the skin-associated bacterial communities (Liston 1957; Colwell 1962; Horsley 1973), only one squirrelfish clone matched most closely to a characterized pseudomonad, although 2 additional clones from the squirrelfish library were closely related to *Burkholderia* spp., a genus that was once part of the *Pseudomonas* (Yabucchi et al. 1992). No close relatives within either genus were found in the lionfish clone library. Of the other genera commonly isolated from fish skin, including *Achromobacter*, *Moraxella*, *Acinetobacter*, and *Vibrio* (Colwell 1962; Horsley 1973; Gilmour et al. 1976; Austin 1983), only *Vibrio* spp. and *Achromobacter* spp. were represented in our clone libraries. Relatively few clones most closely related to *Vibrio* spp. were recovered from lionfish and squirrelfish libraries, and *Achromobacter* spp. were only observed in the squirrelfish clone library. Other genera prevalent in our clone libraries but not commonly reported from cultivation-based studies include *Alteromonas*, *Pseudoalteromonas*, *Marinomonas*, *Nonlabens*, *Photobacterium*, and *Serratia*. Using denaturing gradient gel electrophoresis methods to separate amplified portions of the 16S rRNA gene, Jensen et al. (2004) also recovered members of the *Marinomonas* and *Photobacterium* from reared larval halibut, suggesting that they are common members of fish-associated microflora. It is now generally accepted that culture-dependent techniques recover only a small percentage of bacteria from environmental samples, indicating that only a small fraction of the resident organisms was likely examined in the early studies of fish skin microflora. In 1992, based on total counts that were conducted with various microscopy methods after DAPI staining, Bernadsky and Rosenberg (1992) calculated that they were able to isolate only 0.01 % of the bacteria present on cornetfish skin. Thus, the difference in methods employed in this and other recent molecular-based studies compared to the culture-dependent studies performed previously is likely responsible for the disparity in results obtained.

One of the genera detected in both clone libraries was *Pseudoalteromonas,* occurring more frequently in lionfish (14.9 % of sequences) than in squirrelfish (3 % of sequences). Members of this genus are exclusive to the marine environment, commonly associated with eukaryotic hosts, and well known for the production of biologically active metabolites (Holmström and Kjelleberg 1999). The production of anti-bacterial metabolites is thought to aid in the colonization of surfaces, including host surfaces. In 1997, Maeda et al. (1997) used an anti-microbial producing strain of *Pseudoalteromonas* to repress the growth of deleterious bacteria and viruses and improve the growth of farmed fish. Thus, the presence of numerous clones with sequences most closely related to *Pseudoalteromonas* spp. suggests a potential role in providing protection from pathogens for the host fishes. Additionally, fishes with diverse bacterial communities have been linked to elevated disease resistance (Chabrillón et al. 2005). These microorganisms may serve as probiotics by interfering with the attachment of pathogens, either by directly inhibiting the growth and proliferation of pathogens or by filling host niches and blocking attachment of these pathogens (Nikoskelainen et al. 2001).

Marine animals have been shown to acquire their associated microflora via two main mechanisms. In some cases, microorganisms are transferred vertically with the gametes from parents to offspring. This has been demonstrated for some marine sponges (e.g., Usher et al. 2001; Enticknap et al. 2006; Sharp et al. 2007) and bivalves (e.g., Cary 1994; Krueger et al. 1996). In other animals, their associated microbial communities are acquired laterally from the ambient environment. Because lionfish eggs do not appear to be colonized with microorganisms, one may infer that lionfish obtain their bacterial communities from their environment. However, the eggs in this project were collected directly from the ovaries of female fishes, and the potential for introduction of bacteria during expulsion from the fish or from sperm at fertilization was not assessed. If lionfish acquire their associated bacteria from their environment, it minimizes the possibility of introducing novel microorganisms to the invaded range.

Previous research has clearly shown the negative impact of the invasive Indo-Pacific lionfish on coral reefs of the US Atlantic Coast, Caribbean Sea, and the Gulf of Mexico (Albins and Hixon 2008; Morris and Akins 2009; Barbour et al. 2010). However, the potential roles of the microbial communities associated with the fish have not been previously addressed. Bacteria serve as pathogens of marine organisms and as symbionts that aid in performing important ecological and physiological functions (Cahill 1990; Bernadsky and Rosenberg 1992; Austin 2002). This broad range of potential interactions with their hosts highlights the importance of investigating the bacterial communities associated with invasive organisms. The results presented here indicate that the invasive lionfish support a different bacterial community than native fishes. While not definitive, the bacterial communities associated with the invasive lionfish appear to be derived from the environment and do not contain known fish pathogens, whereas the bacteria associated with the native fishes included several potential pathogens. Ongoing studies in our laboratory include examinations of the bacterial communities on fish from both the invaded and native ranges, and analyses of potential symbiotic interactions between lionfish bacteria and the host fish.

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