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Black Band Disease Microbial Community Variation on Corals in Three Regions of the Wider Caribbean

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Abstract

Black band disease (BBD) is a pathogenic consortium of microorganisms that primarily affects massive framework-building scleractinian corals on reefs worldwide. There has been considerable debate concerning the microbial community composition of BBD. The aim of this study was to utilize microbial profiling to assess overall patterns of variation in the BBD bacterial community with respect to geographic location, host coral species, time, and nutrient regime. Length heterogeneity polymerase chain reaction (LH-PCR) was employed to differentiate BBD communities based on the natural variation in the sequence lengths within hypervariable domains of the 16S rRNA gene. Analysis of LH-PCR profiles of 97 BBD samples using multivariate ordination methods and analysis of similarity revealed significant clustering with respect to geographic region when comparing BBD sampled from reefs near Lee Stocking Island in the Bahamas' Exuma Chain, the Northern Florida Keys (NFK), and St. John in the US Virgin Islands. There was much variability in BBD community composition on a regional basis, between sites in the NFK, and in terms of coral host species. The observed differences among BBD microbial community profiles were driven primarily by variation in relative abundance of 313–316-bp amplicons, which correspond to cyanobacteria and α-proteobacteria. The results obtained in this study support previous reports of intrinsic variability and complexity of the BBD microbial community but also suggest that this variability has biogeographic patterns.

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Introduction

The dramatic decline of coral reefs over the past three decades [14, 20] has led to much attention and research concerning processes that potentially drive reef degradation. Numerous natural and anthropogenic factors have been implicated in the observed coral loss [15, 27], including infectious diseases of scleractinian corals [28]. Concurrent with the decline of coral reefs, the number of coral diseases, coral species infected, reported cases, and the geographic distribution of diseases have all dramatically increased [12, 21, 24, 32, 39]. Despite the documented and potential impacts on coral reef health and increased attention in this field, relatively little is known regarding the etiology of many coral diseases [24].

Black band disease (BBD), the first reported coral disease [1], is the most widely distributed coral disease affecting reefs worldwide [32]. At least 64 scleractinian coral species are susceptible to BBD, and of these, 19 are found in the Caribbean [32]. On Caribbean reefs, BBD primarily infects massive framework-building scleractians including *Colpophyllia natans*, *Diploria labyrinthiformis*, *Montastraea annularis*, *Montastraea cavernosa*, *Montastraea faveolata*, and *Siderastrea siderea* [7, 17, 30, 37].

Black band disease is a polymicrobial disease that migrates across an infected coral colony, destroying coral tissue and leaving behind bare limestone skeleton [4]. Infections appear as a darkly pigmented mat, or band, which can be several millimeters to several centimeters wide and approximately 1 mm thick. The visually dominant members of the BBD consortium are gliding,

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filamentous, nonheterocystous cyanobacteria [29]; gliding, filamentous sulfide-oxidizing bacteria [4, 11, 23]; and numerous small rods and cocci representing at least 50 heterotrophic bacterial species [6, 10, 31].

While a number of studies have focused on identifying and characterizing individual BBD microorganisms using both cultivative and noncultivative techniques [6, 8, 10, 36], two studies have looked at the whole-community BBD samples using molecular profiling [6, 9]. Cooney and colleagues identified variability in 11 BBD samples collected from Caribbean corals using amplified ribosomal DNA restriction analysis and denaturing gradient gel electrophoresis [6]. On the other hand, Frias-Lopez and co-workers observed similar profiles among 10 BBD samples collected in Curaçao using terminal restriction fragment length polymorphism (TRFLP) [9]. TRFLP profiles from BBD samples collected in New Britain (as well as a BBD from an infection observed in an aquarium) were significantly different from the Curaçaoan profiles [9].

In this study we examined the BBD community composition of a large number of BBD samples (97) using an alternate approach: amplicon length heterogeneity polymerase chain reaction (LH-PCR). Performed on whole-community DNA, LH-PCR differentiates organisms based on the natural variation in the sequence lengths of hypervariable domains within 16S rRNA genes, generating profiles that indicate the relative abundance of amplicons present [34]. These amplicons generally indicate phylogenetic differences; however, it is possible for multiple, distantly related organisms to give rise to amplicons of identical base pair (bp) lengths but composed of different base sequences [19]. Recently, LH-PCR has proven useful in profiling microbial communities and in various monitoring efforts [2, 18, 19, 34, 35]. For this study, we analyzed BBD samples collected from multiple coral reefs across three regions in the Caribbean [the Bahamas' Exuma Chain, the Florida Keys, and St. John in the US Virgin Islands (USVI)], including nine coral host species, samples from the same infection over time, and those collected in conjunction with an *in situ* nutrient dosing experiment.

Methods

Sample Collection. Black band disease-infected coral colonies were identified during surveys as part of multiyear monitoring programs near Lee Stocking Island (LSI) in the Bahamas Exuma Chain, on the Northern Florida Keys (NFK) reef tract, and near St. John in the USVI. In this study, 19 reef sites were sampled among these three geographic regions. Samples of BBD near LSI were collected on July 13–25, 2004, from seven reef sites (Fig. 1, B). Big Point, Rainbow Gardens, and Tug and Barge are shallow (1–5 m), protected patch reefs dominated by large boulder corals and subject to strong tidal influences. Whitehorse is a well-demarcated spur and groove reef formation with depth ranging from 3 to 13 m. Horseshoe, North Perry, and South Perry are complex fringing reefs with moderate wave action and 4–20-m depths. These three sites, along with Whitehorse, are exposed to the deepwater Exuma Sound and potential cool water and nutrient upwelling. Black band disease samples were also collected in the NFK during June 14–19, 2004, and May 16–20, 2005, from nine reef sites: Carysfort South, Conch Shallow, Davis Ledge, French, Grecian Rocks, Key Largo Dry Rocks, Molasses, Watson, and White Banks (Fig. 1, A). All of these sites are in patch reefs that lie along the reef tract in approximately 2–6 m of water. Black band disease samples were collected on September 6, 2005, from fringing reefs (2–5 m) at Haulover Bay, Hawknest, and Watermelon Cay off St. John in the USVI (Fig. 1, C). A total of nine host coral species were sampled: *C. natans*, *Dichocoenia stokesi*, *Diploria labyrithiformis*, *Diploria strigosa*, *M. annularis*, *M. cavernosa*, *M. faveolata*, *Meandrina meandrites*, and *S. siderea*.

Duplicate BBD samples were collected from each infected coral colony on the LSI and NFK reefs using sterile 10-mL syringes while SCUBA diving. Only individual samples were collected on the USVI reefs. After the BBD sample was drawn into the syringe, it was allowed to clump before decanting approximately 8 mL of seawater from the syringe. The BBD samples were stored in the dark on ice (no longer than 4 h) until processing at the field laboratory, where the BBD samples were aseptically transferred into sterile, 2-mL cryovials and frozen. All samples were stored at −20°C until DNA extraction.

Nutrient Dosing and Sampling Over Time. Variation in the BBD microbial community was also assessed over time, and in response to *in situ* experimental nutrient dosing, for 10 naturally occurring BBD infections on *S. siderea* on Horseshoe reef near LSI. Nutrient concentrations near experimental colonies ($n = 5$) were increased using 15 g Osmocote™ 9-6-12 time release fertilizer (Scotts, Maryville, OH, USA) in diffusive nylon bags attached to bare coral skeleton 5–10 cm behind the advancing BBD line. Empty nylon bags were used for the control colonies ($n = 5$) exposed only to ambient nutrient concentrations. The fertilizer-filled bags were replaced every 5 days to maintain elevated nutrient concentrations over the duration of the experiment (see Voss and Richardson [38] for detailed methods). The BBD infections on all colonies were sampled at 5-day intervals from July 6 to 25, 2005, using the methods described above.

DNA Extraction and PCR Conditions. The Fast DNA® SPIN Kit for Soil (QBiogene, Vista, CA, USA), with slightly modified protocols [19], was used to extract whole-community genomic DNA from the BBD samples,

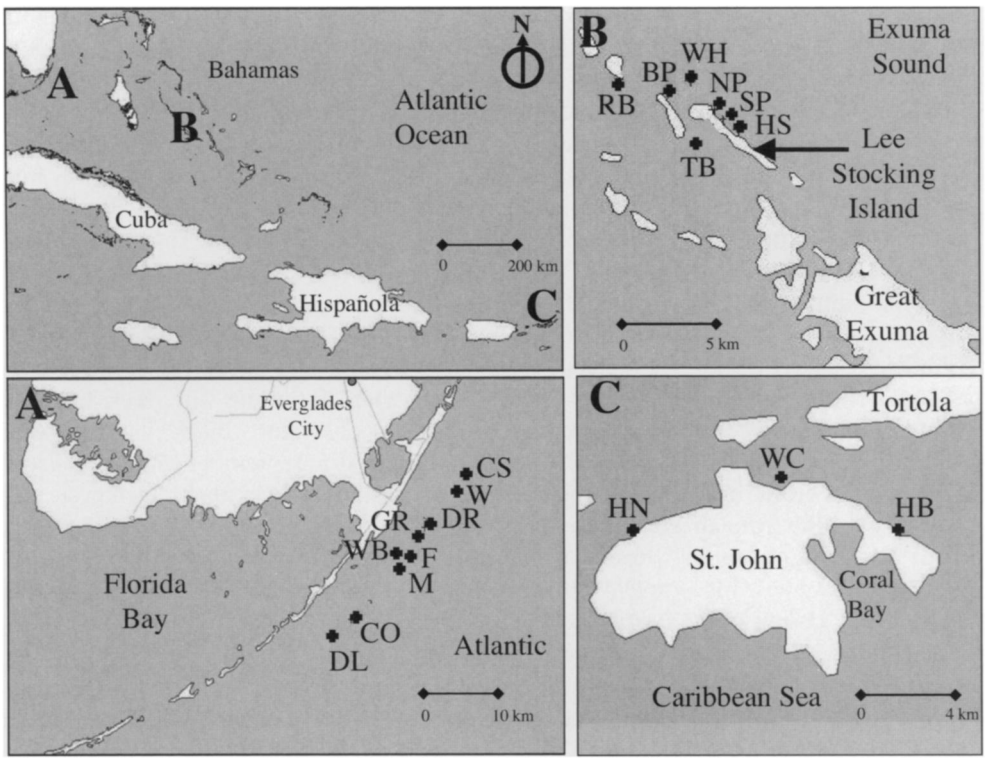

Figure 1. Black band disease samples were collected from study reefs in the NFK (*A*), near LSI in the Bahamas's Exuma Chain (*B*), and on the north side of St. John in the USVI (*C*). Reef locations are represented by *labeled crosses*. NFK: *CS* Carysfort South, *CO* Conch, *DL* Davis Ledge, *DR* Key Largo Dry Rocks, *F* French, *GR* Grecian Rocks, *M* Molasses, *W* Watson, *WB* White Bank. LSI: *BP* Big Point, *HS* Horseshoe, *NP* North Perry, *RB* Rainbow, *SP* South Perry, *TB* Tug and Barge, *WH* White Horse. USVI: *HB* Haulover Bay, *HN* Hawksnest Bay, *WC* Watermelon Cay. Note different scales on panels.

as well as from BBD and non-BBD cyanobacteria cultured from BBD samples collected in LSI and the NFK. The genomic DNA extracts were verified using 1% Tris–borate–EDTA (TBE) agarose yield gels and subsequently quantified using Picogreen dye on a Bio-Rad VersaFluor™ fluorometer (Richmond, CA, USA).

The PCR primers used in this study were designed to amplify the domain of the 16S rRNA gene that included hypervariable domains V1 + V2. The fluorescently labeled forward primer 27F-6-FAM (5′-6-FAM-AGA GTT TGA TCM TGG CTC AG-3′) was used with the non-fluorescent reverse primer, 355R (5′-GGT GCC TCC CGT AGG AGT-3′). The final concentrations of the PCR reactions were: 1×PCR buffer, 2.5 mM $MgCl_2$, 0.25 mM of each diethylnitrophenyl thiophosphate, 0.5 μM forward and reverse primer, 0.25 U AmpliTaq® Gold LD DNA polymerase (PerkinElmer, Wellesley, MA, USA), 0.1% bovine serum albumin, fraction V (Sigma-Aldrich, St. Louis, MO, USA), 1 ng genomic DNA, and diethyl pyrocarbonate-treated water, for a final volume of 20 μL. The PCR reactions were carried out in a Peltier Thermal Cycler (PTC-200, MJ Research, Waltham, MA, USA) under the following run conditions: 94°C for 11 min, 25 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 10 min. Cycling parameters were optimized to address template reannealing [33] and kinetic biases [34]. All PCRs were run in duplicate and PCR products were verified with 1% TBE agarose yield gels.

Electrophoresis and Analysis. All samples were denatured by adding 9.5 μl of 96:1 Hi-Di® deionized formamide: GeneScan® ROX 500 standard solution [Applied Biosystems (ABI), Foster City, CA, USA] to 0.5 μl of each PCR product. The PCR products were then separated on a capillary electrophoresis ABI 310 DNA genetic analyzer using filter set D and module DS-30. Injection time was set to 5 s and run time to 28 min.

LH-PCR electropherograms (profiles) from each sample were analyzed using ABI's Prism GeneMapper® research version 3.7 software. The analysis parameters were set to the Local Southern Method and peak amplitude threshold was set at 50 fluorescent units. Profiles were also generated from three separate 2-mL (non-BBD) seawater samples. No peaks greater than 50 units were detected, indicating that seawater bacteria associated with our sampling protocol were unlikely to contribute to the LH-PCR profiles of BBD communities. To increase resolution, no peak correction or smoothing was applied. Analysis was limited to fragments 300 to 400 bp in length. The size (to nearest bp) and relative abundance (i.e., relative fluorescence intensity) of each peak were determined for each profile. Amplicons that comprised less than 0.5% of total relative abundance were eliminated from the analysis. The relative abundance data were averaged among replicates and square root transformed before calculating Bray–Curtis similarity matrices [3].

Nonmetric multidimensional scaling (MDS) [16] was used to visualize differences in the LH-PCR profiles of the

samples. Each ordination was run with 20 random starting configurations to determine the best-fit model for nonparametric regression between the distance among samples in the plot and the Bray–Curtis similarity matrix. Dimensions were selected to minimize final stress, a goodness-of-fit measure related to the relationship between original p-dimensional space and the dissimilarity within the new ordination space. Variations among profiles from different geographic regions and different host species were tested using an analysis of similarity (ANOSIM), a robust analysis method requiring neither normally distributed abundance data nor balanced replicates between groups [5]. Similarity percentage (SIMPER) [5] was used to determine the relative contribution of each amplicon to the average dissimilarity between the different groups. Only those amplicons that contributed >5% to the dissimilarity are discussed. Nonmetric MDS ordinations, ANOSIM, and SIMPER were conducted using Primer 5 (Primer-E, Plymouth, UK).

Although relatively precise amplicon size calling was observed in the data set (maximum SE of 0.3 bp), LH-PCR profiles were also analyzed using the strategy described by Hewson and Fuhrman [13] to test for any potential effects arising from imprecision in amplicon size calling. Using this method, the starting size of 2-bp-wide-bin window frames was shifted by 1 bp over two frames and similarities were calculated for each frame. The maximum similarity between each pair of samples was used for comparisons among community profiles. This technique is a theoretically more conservative approach that reduces the probability of type 1 error when testing for statistical differences among samples. Two-way crossed ANOSIM (region, host species) analysis and pairwise tests were repeated using the maximum similarity matrix for comparison to the standard 1-bp resolution analysis.

Results

Reproducibility. LH-PCR profiles produced from subsamples of the same BBD sample were reproducible both in terms of triplicate samples analyzed and duplicate PCRs. This reproducibility can be seen when viewing the raw data as shown in Fig. 2, i.e., electropherograms, which indicate both the abundance of amplicons (relative peak height) and number of bps (amplicon length).

Variability in BBD Profiles. Comparison of LH-PCR profiles revealed much variability in BBD microbial communities among the different samples from the NFK, LSI, and the USVI and among different coral host species. Representative profiles from each geographic region, sampled from two host coral species, are shown in Fig. 3. In this figure, unlike Fig. 2 which shows raw data, individual peaks were binned with 1-bp resolution, and any overlapping peaks were resolved (see "Methods" section). In the BBD profile from the NFK (on *M. annularis*), 13 amplicons were present, with lengths ranging from 304 to 360 bp, whereas BBD profiles from *S. siderea* at both LSI and the USVI revealed 14 amplicons from 304 to 359 bp and 12 amplicons from 306 to 358 bp, respectively. In addition to the differences in total numbers of amplicons, the relative abundances (peak height) of amplicons among these three samples were highly variable with respect to geographic region.

In this study, 97 BBD samples were analyzed (not including time series/nutrient dosing experiment samples) to assess regional and coral host species variability. Within this data set, a total of 48 amplicons of different lengths were resolved ranging from 301 to 370 bp. Of these, 45 amplicons were present in the NFK samples, whereas 44 and 24 amplicons were observed in the LSI and USVI samples, respectively. There were 21 amplicons common to all three geographic regions, 42 common to the NFK and LSI, 23 common to NFK and USVI, and 21 common to LSI and USVI (the same 21 common to all regions). Each geographic region had unique amplicons: 301 and 318 bp in LSI, 325 bp in NFK, and 306 bp in USVI. While the unique amplicons in the NFK and LSI were relatively rare (and not observed in the representative samples shown in Fig. 3), the 306-bp amplicon was present in all profiles from the USVI.

Multidimensional Comparison by Geographic Regions. Nonmetric MDS and ANOSIM analysis of the LH-PCR profiles indicated significant clustering with

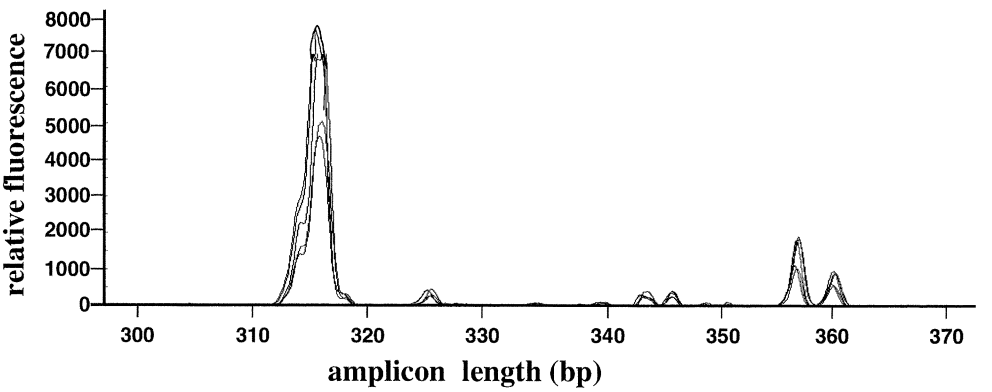

Figure 2. Six superimposed LH-PCR electropherograms from triplicate BBD samples and independent PCRs. The low variability in peak presence and amplitude demonstrate the reproducibility of this technique.

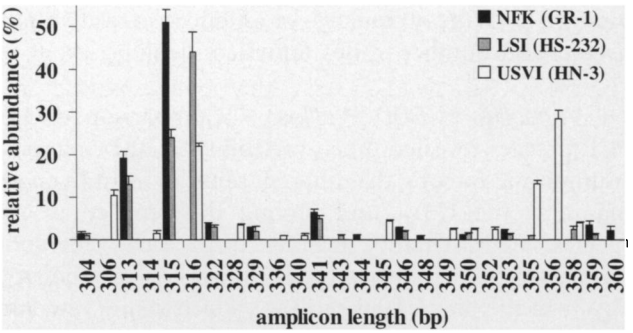

Figure 3. Representative LH-PCR profiles: NFK, Grecian Rocks (BBD on *M. meandrites*); LSI, Horseshoe (BBD on *S. siderea*); USVI, Hawksnest Bay (BBD on *S. siderea*). Means and standard error are shown for the NFK and LSI sample based on four profiles from duplicate extractions and duplicate PCRs. For the USVI, duplicates were not available; mean and SE based on triplicate PCR.

respect to geographic region [Fig. 4A, two-way crossed ANOSIM (region, host species) global $R = 0.501$, $p < 0.01$]. While some overlap is apparent among the three regions, in pairwise comparisons, all regions were statistically discriminant from one another (NFK:LSI, $r = 0.473$, $p < 0.01$; NFK:USVI, $r = 0.931$, $p < 0.01$; LSI:USVI, $r = 0.574$, $p < 0.05$), suggesting that unique bacterial assemblages are associated with BBD in each of these three geographical areas.

SIMPER analyses identified the relative contributions of each amplicon to average dissimilarity of samples between regions (Table 1). LH-PCR profiles from BBD collected in the NFK were primarily differentiated from those at LSI by the greater relative abundance of amplicons 315 and 338 bp and lesser abundance of the 313- and 316-bp amplicons (Table 1a). Of these amplicons, those with 315, 316, and 313 bp were the most important, accounting for 21.4, 16.0, and 12.5%, respectively, of the average dissimilarity. Differences in NFK profiles from samples in USVI were driven by greater abundance of the 315-bp amplicon and relatively lesser abundance of 355-, 356-, 316-, and 306-bp amplicons (Table 1b). When comparing LSI to USVI, 313–316-bp amplicons were again more

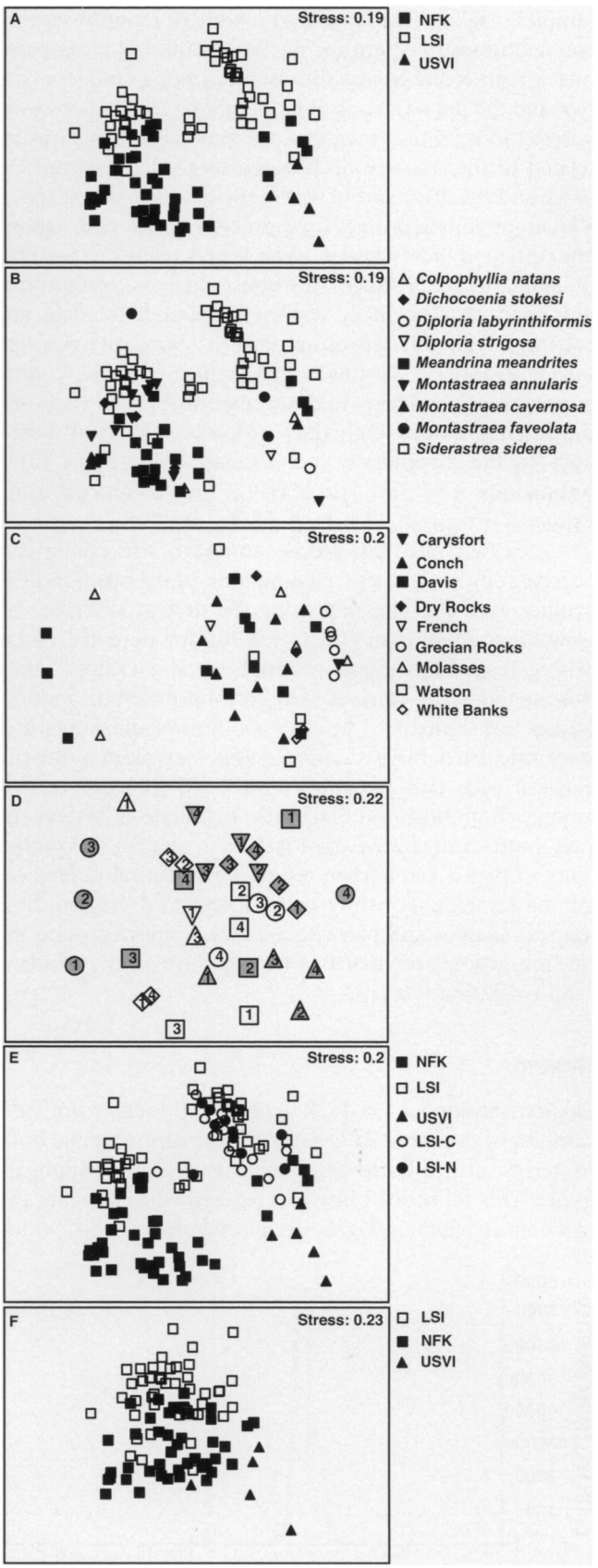

Figure 4. Nonmetric MDS ordinations of LH-PCR profiles from BBD samples. Distance between samples represents similarity in LH-PCR profiles. Stress in each plot is a goodness-of-fit measure between the plot and the original p-dimensional matrix. ***A*** Samples labeled by region. ***B*** Samples labeled by host coral species. ***C*** Northern Florida Keys samples labeled by reef site. ***D*** Samples from the time series/nutrient experiment. In panel ***D***, *shaded symbols* represent control colonies and *open symbols* indicate those colonies exposed to elevated nutrients. The *numbers* within the *symbols* indicated each 5-day interval (i.e., 1=5 days, 2=10 days, etc). The *shapes* represent individual *S. siderea* colonies in each treatment. ***E*** Samples from all regions, including those from time series/nutrient loading experiment. ***F*** Samples analyzed using frame shift method and labeled by region.

abundant at LSI, whereas 306-, 355-, and 356-bp amplicons were more abundant in the USVI (Table 1c). The 355- and 356-bp amplicons were commonly dominant in the USVI samples, and 306-bp amplicons were found exclusively in this region (e.g., Fig. 3). Notably, the 315-bp amplicon was absent from the USVI profiles.

Multidimensional Comparison by Host Coral Species. Multidimensional scaling and ANOSIM analyses also indicated that LH-PCR profiles of BBD microorganisms differed with respect to host coral species [Fig. 4B, two-way crossed ANOSIM (region, host species) global $R=0.184$, $p<0.05$]. Of the nine coral host species investigated, pairwise comparisons indicated that five pairs of host species could be discriminated from one another (Table 2). These were *M. annularis*: *M. cavernosa*, *M. annularis*: *M. faveolata*, *M. annularis*: *S. siderea*, *M. faveolata*: *M. meandrites*, and *M. faveolata*: *S. siderea*.

Multidimensional Comparisons Within Geographic Regions. To assess variation in BBD microbial communities at smaller spatial scales, MDS and ANOSIM analyses were conducted within each geographic region. Within the NFK region, LH-PCR profiles differed significantly with respect to site [Fig. 4C, two-way crossed ANOSIM (site, host species) global $R=0.147$, $p<0.05$] but not by host coral species (MDS not shown). Pairwise comparisons indicated six pairs of sites that differed significantly from one another (Table 2). Five samples, three from Davis Reef and two from Molasses Reef, were notably separate from the remaining NFK samples in the MDS plot (Fig. 4C). These five samples were similar to samples from LSI in that they were dominated by the 316-bp amplicon. No significant differences were observed in two-way crossed ANOSIM test with respect to site and host coral species in either LSI or USVI.

Variation Over Time in Control and Nutrient-dosed BBD Infections. The LH-PCR profiles of samples from BBD infections on *S. siderea* at Horseshoe Reef in LSI collected at 5-day intervals demonstrated variability but did not reveal any significant patterns over the 20-day duration of the experiment (Fig. 4D). Furthermore, there were no detectable trends or changes in the BBD microbial community profiles after artificial increases in nutrient availability. Despite temporal variation and nutrient dosing, profiles from these samples remained similar to the other BBD infections in LSI as demonstrated by MDS ordination (Fig. 4E).

Comparison to Frame Shift Analysis Method. Use of the analysis strategy described by Hewson and Fuhrman [13] resulted in similar MDS patterns (Fig. 4F) and increased discrimination among samples with respect to both region and host coral species. As in the standard analysis, profiles differed significantly by both region and coral host species (two-way crossed ANOSIM, region: global $R=0.607$, $p<0.001$, species: global $R=0.381$,

Table 1. Results of SIMPER analyses for significant ANOSIM contrasts between BBD community profiles by region

Amplicon length (bp)	*Mean relative abundance*[x]	*Mean relative abundance*[x]	*%*[y]	*% cumulative*[z]
a				
	NFK	LSI		
315	41	18	21.4	21.4
316	3	25	16.0	37.4
313	4	21	12.5	49.9
338	8	0	5.2	55.1
b				
	NFK	USVI		
315	41	0	23.5	23.5
355	2	26	13.6	37.1
356	4	23	11.5	48.6
316	3	19	10.6	59.2
306	0	11	6.1	65.3
c				
	LSI	USVI		
355	1	26	14.9	14.9
356	0	23	13.4	28.3
316	25	19	13.4	41.7
313	21	1	12.1	53.9
315	18	0	10.6	64.5
306	0	11	6.4	70.9

a, NFK vs LSI; b, NFK vs USVI; c, LSI vs USVI.
[x]Mean relative abundance of each amplicon as a percentage of total amplicon abundance.
[y]Amplicon contribution as a percentage dissimilarity between the two groups. Lists are truncated to include only those amplicons that contribute >5% to the differences between groups.
[z]Cumulative contributions of amplicons to dissimilarity between groups.

Table 2. Comparison of BBD samples collected from different reefs and host coral species in the NFK, LSI, and USVI

	Host coral species and number of samples								
Reef Site	*Cn*	*Dsi*	*Dl*	*Dst*	*Ma*[a,b,c]	*Mc*[b]	*Mf*[c,d,e]	*Mm*[d]	*Ss*[a,e]
NFK									
Conch[u,v,w]								9	
Carysfort					1				
Davis								12	
Dry Rocks[x]					2				
French	1								
Grecian Rocks[u,x,y,z]					5				
Molasses[v,y]		2					2		
Watson[w,z]									4
White Bank						3			
LSI									
Big Point				1					2
Horseshoe					1				31
North Perry									2
Rainbow							1		3
South Perry	2								2
Tug & Barge									3
White Horse									3
USVI									
Hawksnest Bay									1
Haulover Bay			1		1	1			
Watermelon Cay				1					

The number of samples collected for each site and host species are shown. Significant differences in pairwise ANOSIM comparison between host coral species (among all regions) and reefs sites in the NFK are indicated by pairs with the same superscript letter.

Cn = *C. natans*, Dsi = *D. stokesi*, Dl = *D. labyrinthiformis*, Dst = *D. strigosa*, Ma = *M. annularis*, Mc = *M. cavernosa*, Mf = *M. faveolata*, Mm = *M. meandrites*, Ss = *S. siderea*

$p<0.005$). The frame shift analysis method resulted in global R values greater than those obtained by 1-bp resolution analysis. Increased R values were also observed in pairwise comparisons by region (NFK:LSI, $R=0.578$, $p<0.01$; NFK:USVI, $R=0.839$, $p<0.05$; LSI:USVI, $R=0.716$, $p<0.05$). Finally, while standard analysis methods identified five pairs of host species that were significantly different from one another (Table 2), frame shift analysis discriminated two additional pairs (*M. annularis*: *M. meandrites* and *M. cavernosa*: *M. meandrites*).

Discussion

LH-PCR profiling revealed that distinct microbial assemblages are associated with BBD infections in three different regions of the wider Caribbean: the NFK, LSI, and USVI. Geographically distinct BBD microbial communities have been reported previously based on TRFLP community profiling; however, this study compared samples from the Indo-Pacific and the Caribbean [9]. The TRFLP profiles for BBD samples collected from one *M. annularis* and nine *D. strigosa* colonies in Curaçao were the same but differed significantly from BBD samples collected on *Porites lutea* in Papua New Guinea [9]. In contrast, the data reported here indicate variability among BBD microbial assemblages on conspecific coral hosts at the same reef. Furthermore, the patterns of spatial variability in the BBD microbial community exhibited finer geographic resolution. In addition to distinguishing between BBD communities in different regions of the wider Caribbean, significant spatial patterns were also apparent at a much smaller scale when comparing BBD profiles from various reef sites in the NFK (Fig. 4C).

LH-PCR profiles of the BBD microbial assemblage also differed with respect to host coral species. Interpretation of observed patterns in terms of the host species is complex, however, because this variable can be confounded with geographic location. For example, BBD prevalence can vary among individual coral species in different locations [37], preventing optimal sampling designs. Nonetheless, geographic location (and, most likely, the associated environmental drivers in each locale) appears to be more important than host species in structuring BBD communities. We found that BBD samples from five different coral host species in the USVI were more similar to one another than to samples from conspecific coral hosts in LSI or the NFK. Previous findings, which compared two coral species, indicated similar BBD community TRFLP profiles in one geographic region [9]. In apparently healthy corals, distinct microbial communities have also been identified on different coral species [26].

While the results of this study demonstrate that BBD communities are dynamic over relatively short time scales (Fig. 4D, shaded symbols), there were no clear

trends or successional patterns in the relative abundance of various amplicons. However, this experiment was conducted for a period of 3 weeks, much shorter than the months-long duration of most BBD infections on LSI and NFK reefs; furthermore, BBD is active year-round on USVI reefs (Kaczmarsky, personal communication). Nonetheless, the temporal variability we observed has important implications regarding the community structure of BBD in that the sampling time point may influence both community profiling and clone library results. Time series samples in this study remained clustered among other samples from LSI (Fig. 4E). Furthermore, although experimental increases in nutrient availability caused accelerated rates of BBD disease progression and host coral tissue loss [38], there was no significant observable effect on the structure of the BBD microbial communities (Fig. 4D, open symbols).

When comparing the standard analysis method with 1-bp resolution to the frame shift strategy suggested by Hewson and Fuhrman [13], increased discriminatory power was observed among *a priori* sample groups. The frame shift strategy, using maximum similarity between samples, is designed to more conservatively address the null hypothesis that microbial communities do not differ among samples. In the BBD community profile data set, use of frame shift analysis increased similarity within each sample group. As a result, greater ANOSIM global *R* values implied higher levels of dissimilarity between groups. While the frame shift strategy may be highly effective in data sets where imprecise size calling is apparent, in certain cases, such as that described here, the method may understate community variation within *a priori* groups. Furthermore, use of the frame shift strategy precludes SIMPER analysis and its elucidation of the relative contributions each amplicon makes to dissimilarity between groups. In this study, LH-PCR was a rapid, economical, reproducible, and accurate method for describing overall patterns of variation and change in microbial communities. However, this profiling technique is limited in its ability to identifying individual species. As suggested by Hewson and Fuhrman, imprecise primers or template DNA in other LH-PCR studies may warrant frame-shift analyses.

The SIMPER analyses revealed that the differences observed in BBD community profiles between regions were driven primarily by variation in amplicons 306, 313, 315, 316, 355, and 356 bp in length. Amplicons of 313 and 316 bp were abundant at LSI, 315-bp amplicons were more common in NFK samples, and USVI profiles were dominated by 306-, 316-, 355-, and 356-bp amplicons. There is evidence that the amplicons we observed in the 313–316-bp range are associated with cyanobacteria and alphaproteobacteria. Previous studies using *in silico* analyses of sequences from both bacterial isolates and clones from seawater extractions associated these amplicons with both cyanobacteria and alphaproteobacteria [2, 34]. We have also reported the presence of amplicons ranging from 313 to 316 bp in a study that combined sequence analyses of clones generated from BBD infections on two *S. siderea* colonies in LSI [31]. Using *in silico* analysis, amplicons of this length were associated with sequences homologous to both cyanobacteria and alphaproteobacteria [31]. Furthermore, four BBD cyanobacteria from both the NFK (2) and LSI (2), as well as three non-BBD reef-associated cyanobacteria from the NFK cultured in the laboratory and analyzed separately using LH-PCR, produced amplicons 313–315 bp in length (Table 3). Further examination is needed to identify the species and genera of BBD microbes that are associated with these specific amplicons.

Previous molecular studies have identified multiple cyanobacterial genera associated with BBD infections [6, 8, 9, 10]. Because no single amplicon potentially indicative of cyanobacteria was present in all samples, this study supports the hypothesis that the presence of any one single cyanobacterial species is not required for BBD to occur. Rather, different cyanobacterial species may possess the physiological capabilities required to function in the dynamic chemical environment of the BBD mat [4, 25].

In addition to identifying biogeographic trends in BBD microbial communities, the data presented here help explain the discrepancies between results of different molecular microbial community analyses of BBD [6, 8, 9, 10, 36]. Variability in the structure of the BBD microbial community over relatively small spatial and temporal scales may be responsible for the variation observed in

Table 3. Amplicon lengths associated with BBD and non-BBD cyanobacteria on reefs in LSI and the NFK

Source	*Genus*	*GenBank accession #*	*Amplicon length (bp)*	*Location*
BBD	*Geitlerinema*	DQ151461[a]	315[a]	NFK
	Geitlerinema	EF110974	313	LSI
	Geitlerinema	DQ680351	314	LSI
	Leptolyngbya	EF110975	315	NFK
Non-BBD[b]	*Pseudanabaena*	EF110976	315	NFK
	Leptolyngbya	–	313	NFK
	Leptolyngbya	–	315	NFK

[a]Data from Ragoonath [22].
[b]Samples collected from cyanobacterial mats on sediment patches of healthy (non-BBD) *Dendrogyra cylindricus*.

BBD clone libraries and profiling efforts from different studies. Rather than contradictory, together, these reports may attest to the intrinsic variability and complexity of the BBD microbial community.

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