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Characterization of black band disease in Red Sea stony corals

Orit Barneah,[1] Eitan Ben-Dov,[1,2] Esti Kramarsky-Winter[3,4] and Ariel Kushmaro[1]*

[1]*Department of Biotechnology Engineering, Ben-Gurion University of the Negev, PO Box 653, Be'er-Sheva, 84105, Israel.*

[2]*Achva Academic College MP Shikmim, 79800, Israel.*

[3]*Department of Zoology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Tel Aviv, 69978, Israel.*

[4]*Pacific Biomedical Research Center University of Hawaii at Manoa Honolulu, HI 96822, USA.*

Summary

Microbial communities associated with black band disease (BBD) in massive stony corals from the Northern Red Sea (Eilat) were examined for the first time using molecular tools and microscopy. A high microbial diversity was revealed in the affected tissue in comparison with the healthy area of the same colony. Microscopy revealed the penetration of cyanobacteria into the coral mesoglea and adjacent tissues. Cyanobacterial sequences from Red Sea BBD-affected corals formed a cluster with sequences previously identified from black band and red band diseased corals from the Indo-Pacific and Caribbean. In addition, 11 sequences belonging to the genus *Vibrio* were retrieved. This group was previously documented as pathogenic to corals. Sulfate-reducing bacteria, a group known to be associated with BBD and produce toxic sulfide, were studied using specific primers for the amplification of the dissimilatory sulfite reductase gene (*dsrA*). This technique facilitated and improved the resolution of the study of diversity of this group. All the sequences obtained were closely related to sequences of the genus *Desulfovibrio* and 46% showed high homology to *Desulfovibrio desulfuricans*. The complex nature of BBD and the lack of success in isolating a single causative agent suggest that BBD may be considered a polymicrobial disease.

Received 23 January, 2007; accepted 16 March, 2007. *For correspondence. E-mail arielkus@bgu.ac.il; Tel. (+972) 8 6479024; Fax (+972) 8 6472983.

Introduction

Black band disease (BBD) is a widespread coral disease, which infects mainly the massive-framework-building corals (Frias-Lopez *et al*., 2004). It was first described on reefs of Belize, the Florida Keys and Bermuda (Antonius, 1973; Garrett and Ducklow, 1975). In the following decades it has been also reported to occur in the Indo-Pacific and Red Sea (Antonius, 1985; Al-Moghrabi, 2001) and in the Great Barrier Reef (Dinsdale, 2002; Willis *et al*., 2004), indicating that this disease has a global distribution.

A seasonal pattern of the disease has been reported with higher incidences coinciding with elevated water temperatures (Kuta and Richardson, 2002). Symptoms of the disease are well described and include the black band (5–30 mm wide) that moves across the surface of the coral colony causing tissue lysis leading to either partial or complete death of the colony (Viehman *et al*., 2006). Although this disease affects a relatively low proportion of the susceptible coral community (approximately 1%), its persistence in the environment and rate of progress across colonies (3 mm–1 cm day^{-1}) make it an important threat to the reef community (Antonius, 1981; Edmunds, 1991; Carlton and Richardson, 1995; Kuta and Richardson, 1996).

Black band disease was reported from the Red Sea by Antonius (1988). The disease was encountered on six different locations in the vicinity of Jeddah port, Saudi Arabia and in Al Wajh, which is located further north. The severity of the disease was ranked from rare to moderate and its presence was found to be positively correlated to seawater pollution and temperature. Since the above-mentioned study, only limited data on the occurrence of BBD in this area have been available. In 1996 an unusual BBD outbreak was reported in the northern tip of the Gulf of Aqaba (Al-Moghrabi, 2001). A quantitative survey in this area showed that the number of infected colonies at the Aqaba industrial zone was 10 times higher that at a protected site nearby (Al-Moghrabi, 2001). Despite this, to date, the microbial community associated with BBD in the Red Sea corals has not been studied.

Previous studies showed that similar to other stratified microbial communities (e.g. biofilms and microbial mats), the BBD microbial mat oxygen dynamics was dominated by the photosynthetic activity of the cyanobacterium (for

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review see Carlton and Richardson, 1995). It was found that changes in light intensity caused changes in O_2 concentration, in turn causing an opposite trend in H_2S concentration. This resulted in alterations between oxic and anoxic state inside the BBD mat (Carlton and Richardson, 1995). In spite of the similarities between BBD and other microbial mats, BBD is unique in that the < 1-mm-thick biofilm migrates horizontally across a living animal substratum that is subsequently killed by an anoxic, sulfide-rich microenvironment, created under the band (Carlton and Richardson, 1995). The deadly sulfide is presumably generated by sulfate-reducing bacteria (SRB) (Richardson, 2004). To date only limited information is available regarding this group involvement in the BBD.

Black band disease is believed to be caused by a consortium of microorganisms (Antonius, 1981; Richardson *et al*., 1997; Richardson, 1998; Dinsdale, 2002), and may not have a primary pathogen (Richardson, 2004). The recent progress in molecular techniques has enabled molecular characterization of the bacterial community associated with BBD (Cooney *et al*., 2002; Frias-Lopez *et al*., 2002; 2004; Sekar *et al*., 2006). It was found that the BBD microbial community is dominated, in terms of biomass, by filamentous cyanobacteria (Rützler and Santavy, 1983; Bythell *et al*., 2002; Cooney *et al*., 2002; Frias-Lopez *et al*., 2003; Richardson, 2004), numerous heterotrophic bacteria (Garrett and Ducklow, 1975; Cooney *et al*., 2002), sulfide-oxidizing bacteria (Ducklow and Mitchell, 1979; Viehman and Richardson, 2002), SRB (Chet and Mitchell, 1975; Garrett and Ducklow, 1975; Cooney *et al*., 2002; Frias-Lopez *et al*., 2002; Viehman *et al*., 2006) and marine fungi (Ramos-Flores, 1983). This diversity pattern differed according to geographic region, host coral species and methodology used (Cooney *et al*., 2002; Frias-Lopez *et al*., 2004; Sekar *et al*., 2006). In spite of the fact that this disease was already identified in the 1970s there is still a wide gap of knowledge pertaining to its etiology.

The aim of the present study therefore is to characterize the composition and diversity of the bacteria present in the BBD bacterial mat and in adjacent affected tissues of corals from Eilat (Red Sea) using a diverse set of molecular techniques. To do so we ascertained general bacterial diversity, using traditional 16S rRNA gene primers and inosine-modified 16S primers. In addition, specific primers for the amplification of the dissimilatory sulfite reductase gene (*dsrA*) were used in order to determine the diversity of SRB associated with the BBD mat.

Results

Black band disease in Eilat (Red Sea) was found to affect mainly a number of massive Faviid coral genera including *Favia*, *Favites*, *Goniastrea* and *Platygyra* (Fig. 1). Microscopic observations of BBD mat samples from a *Favia favus* included cyanobacteria filaments (Fig. 2A) that exhibit typical fluorescence of the photosynthetic pigments (orange) with spectrophotometric absorption maximum at 565 nm. This indicated the presence of the light-harvesting cyanobacterial pigment phycoerythrin. Histological sections of the black band and underlying tissues (Fig. 2B and C) revealed an area of necrosis. The area juxtaposed to the band included intact fragments of mesoglea devoid of adjacent cellular layers. Dense populations of filamentous microorganisms morphologically identified as cyanobacteria were found in the degraded tissue. At the interface between the band and apparently healthy tissue, cyanobacteria were found penetrating the coral tissue, in the mesoglea or at the point of attachment of the tissues to the mesoglea. Cells in this area also were necrotic and many pyknotic nuclei are visible (Fig. 2B and C).

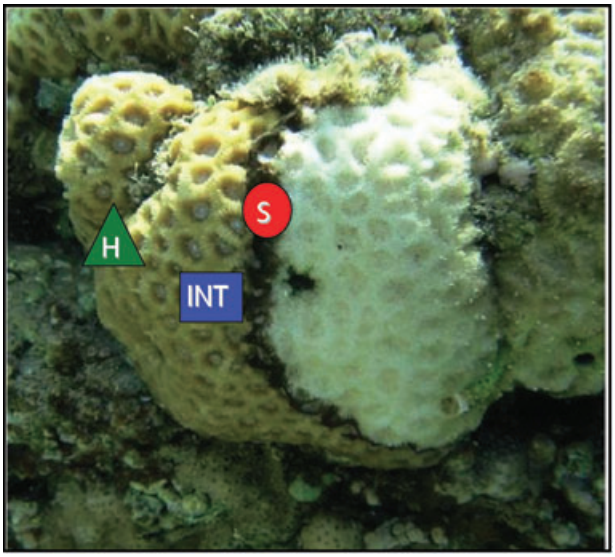

Fig. 1. Black band disease on *Favia* sp. from the Gulf of Eilat. As the band moves across the coral it completely degrades coral tissue and leaves behind exposed coral skeleton. Geometric forms indicate the position of different samples taken: (▲) healthy region (H), (■) interface region (INT), (●) sick region (S).

We analysed the black band community and surface mucus layer from five different diseased Faviid corals, which were sampled at different seasons (see Table 1). During this study a total of 14 16S rRNA gene libraries were constructed (see Table 1) using universal and modified 16S primers, yielding 215 sequences (Accession No. EF089403–EF089533, EF433087–EF433174). Eight libraries of the 16S rRNA gene of the black band mat (S), three from the coral surface mucus layer adjacent to the band (INT) and three from the apparently healthy surface mucus layer (H) were obtained using universal and modified 16S primers. Three additional libraries were obtained from INT and S areas of the corals (see Table 1) using

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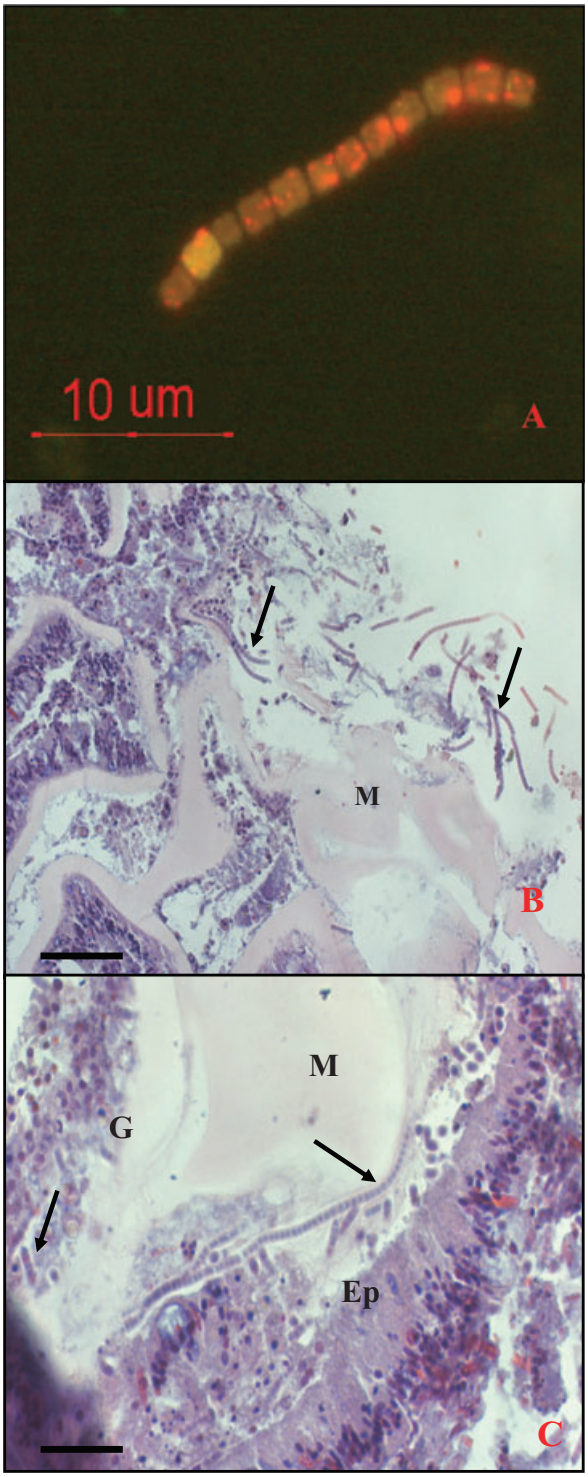

Fig. 2. Black band cyanobacteria from *Favia favus* from the Red Sea.
A. Fluorescence micrograph of a filament. Scale bar = 10 μm.
B and C. Histological section of *F. favus* with BBD. (B) Arrows denote penetration of the cyanobacterial filaments into the coral tissue. Note the mesoglea (M) is stripped of cells. Scale bar = 50 μm. (C) The black band cyanobacterium filament (arrow) can be seen at the base of the epidermis (Ep), gastrodermis (G) and in the mesoglea (M). Scale bar = 30 μm.

the DSR primers, yielding 20 sequences of the *dsrA* gene (Accession No. EF089534–EF089544, EF089549–EF089557).

The diversity of the microbial community of the three sampled regions of BBD corals is presented in Fig. 3. In general, there is a lower diversity at the phylum and division levels, in apparently healthy parts in comparison with INT and S areas of the BBD corals (Shannon–Weiner indices for the three regions were 1.53 for H, 3.20 for INT and 2.85 for S). The healthy regions were dominated by γ-proteobacteria (52%) followed by CAB-I cluster (22%) (Fig. 3A). The intermediate area (INT; Fig. 3B) was also dominated by γ-proteobacteria (30%) followed by actinobacteria (21%) and cyanobacteria (15%). Interestingly there is an appearance of bacteroidetes group (5%) and firmicutes (4%) in the region. β-proteobacteria and lentisphereae were detected solely in the INT region of the corals (Fig. 3B). Libraries from the S region (Fig. 3C) were dominated by α-proteobacteria (30%) followed by cyanobacteria (25%) and bacteroidetes (16%). γ-proteobacteria represented only 12%, a substantial decline between the H and INT regions (Fig. 3A–C). Two additional proteobacterial groups (δ- and ε-proteobacteria) were detected in the S region (Fig. 3C).

Representatives of the sequences from our libraries together with best-matched sequences from the GenBank were used for the construction of phylogenetic trees. For convenience, we present our phylogenetic analysis of the 16S rRNA gene sequences [obtained from polymerase chain reaction (PCR) products using 16S and 16Si primers] in three separate trees: (i) cyanobacteria (Fig. 4), (ii) proteobacteria (Fig. 5), (iii) non-proteobacteria (Fig. 6). In addition, the *dsrA* gene sequences are presented in Fig. 7.

Cyanobacteria

Cyanobacteria were associated with the black band community in August, January and May. No cyanobacteria sequences were detected in the clone library constructed from the November BBD mat sample. Overall, 37 cyanobacterial sequences originating from four diseased corals were obtained (EF089510–EF089533, EF433087–EF433098). The majority of the sequences (31) were found to be highly similar to each other and are represented in our phylogenetic tree by the sequence BB2S16S-9 (Accession No. EF089519) (Fig. 4). Additional five sequences all originating from 'S' and 'INT' libraries cluster together with the above-mentioned group. They also cluster with previously identified cyanobacterial sequences isolated from BBD and red band disease (RBD) corals from the Indo-Pacific (e.g. AY839639–AY839641) and the Caribbean (AY148306). A single cyanobacterial sequence originating from the apparently healthy part of a BBD-affected *Favites*

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Table 1. List of coral samples that were used to construct DNA libraries.

			Samples taken and library names		
Coral species	Sample's name	Date of collection	H	INT	S
Favia sp.	BB	August 2005	ND	ND	BBS16S BBSDSR
Favia sp.	BBD	November 2005	ND	ND	BBDS16S BBDS16Si BBDSDSR
Favia sp.	BB1	January 2006	BB1H16S	BB1INT16S	BB1S16S BB1S16Si
Favites sp.	BB2	January 2006	BB2H16S BB2H16Si	BB2INT16Si	BB2S16S BB2S16Si
Favites sp.	BB3	May 2006	ND	BB3INT16S BB3INTDSR	BB3S16S

H, healthy; INT, interface region; ND, no data; S, sick (band sample).

sp. colony was found related to *Prochlorothrix* group with 91% similarity. The cyanobacterial sequences obtained in this study did not show relatedness to *Geitlerinema* sp. (previously *Phormidium coralliticum*), a cynaobacterium considered previously to be a potential pathogen of BBD in Caribbean corals.

Proteobacteria

The phylogenetic tree presented in Fig. 5 shows that phylum diversity varied among the different regions of the infected colonies. Various sequences obtained in this study were related to sequences previously retrieved from coral environment and specifically from corals, including those affected with BBD. A distinct cluster of 11 sequences closely resembling *Vibrio* species were found in S and INT regions of diseased corals (Fig. 5). The majority (10 out of 11) of these sequences belong to a library that was constructed by using the modified 16S primers (Ben-Dov *et al.*, 2006). In addition, a cluster of *Pseudomonas*-related sequences were obtained from the INT region of the BBD corals (Fig. 5).

Non-proteobacteria

Figure 6 represents sequences retrieved from all regions of the diseased corals. This tree contains representatives from actinobacteria, firmicutes, planctomycetes,

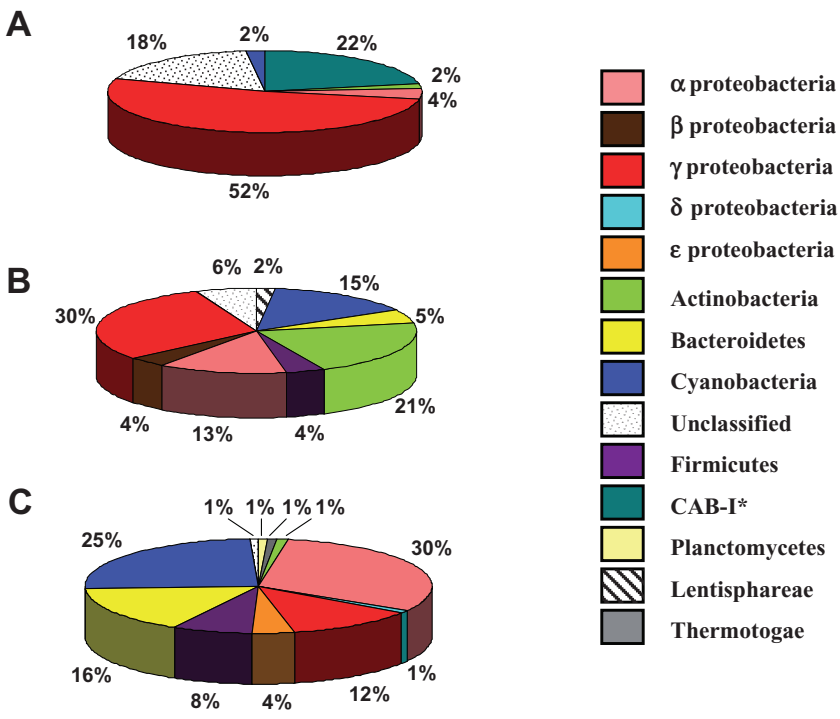

Fig. 3. Division-level diversity of the 16S rRNA gene bacterial sequences obtained from clone libraries constructed with universal and modified 16S primers.
A. Diversity of bacteria retrieved from the surface mucus layer of apparently healthy coral tissue (H) $n = 50$.
B. Diversity of bacteria retrieved from the surface mucus layer adjacent to the black band (INT) $n = 53$.
C. Diversity of bacteria retrieved from the black band (S) $n = 107$.
* CAB-I, uncultured candidate division.

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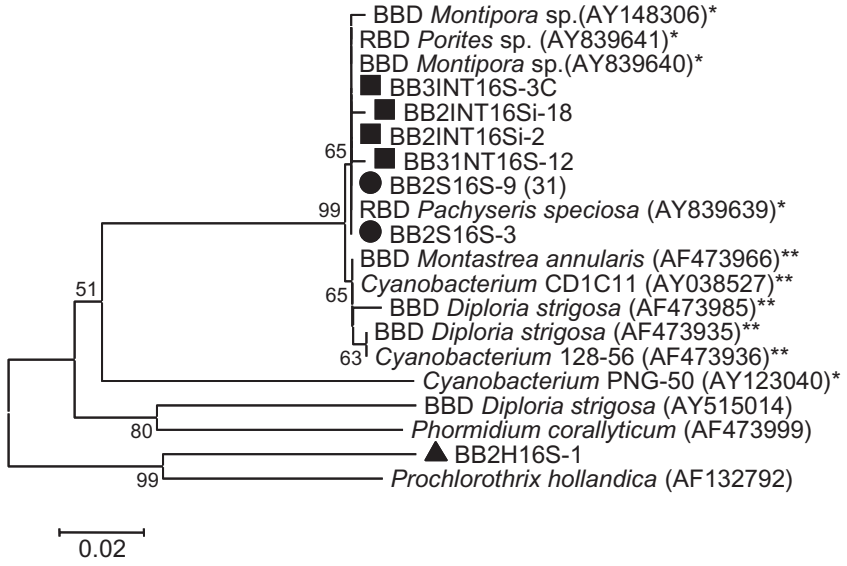

Fig. 4. Neighbour-Joining phylogenetic tree of cyanobacterial 16S rRNA gene sequences from Red Sea BBD together with BBD and RBD (red band disease) sequences from different geographic regions. Sequences from the Indo-Pacific region are marked with '*' and sequences from the Caribbean are marked with '**'. Geometric forms indicate the origin of the sequence according to the sampled area: (●, S) the black band (■, INT) the surface mucus layer adjacent to the band (▲, H) the surface mucus layer of apparently healthy coral tissue. Sequences that were retrieved using the modified 16S primers are indicated by '16Si'. The tree was constructed by the Neighbour-Joining method (Saito and Nei, 1987) with the Mega package (Kumar *et al*., 2004) using partial sequences of 16S rRNA gene. The numbers in parentheses indicate the total number of similar clones on the basis of ≥ 97% identity for each representative sequence. The bar represents two substitutions per 100 nucleotide positions. Bootstrap values (Felsenstein, 1985) are indicated at branch nodes.

bacteroidetes and CAB-I cluster. Cluster CAB-I composed of 19 sequences all originated exclusively from 16Si libraries (using 8F-I and 907R-I primers) constructed from the healthy region of a sampled *Favit*es colony.

Sulfate-reducing bacteria

Sulfate-reducing bacteria were detected in corals sampled in August, November and May (Table 1, Fig. 7). *dsrA* sequences (Accession No. EF089534–EF089544, EF089549–EF089557) originating from three diseased corals, from S or INT regions (Table 1, Fig. 7). Attempts to amplify the *dsrA* gene from samples of surface mucus layer of the healthy coral tissue were not successful. All the sequences obtained were closely related to sequences of the genus *Desulfovibrio*. Eleven of the sequences were found to be highly similar (97–99%) to *Desulfovibrio desulfuricans* (Accession No. CP000112). Nine of the sequences were found closely related to several other *Desulfovibrio* species including *D. longus* (AB061540), *D. simplex* (AB061541), *D. aespoeensis* (AF492838) and *D. desulfuricans* ssp. (AJ289157), but with relatively low similarity values (87–93%).

Discussion

Microorganisms of BBD-affected Faviid corals from the Red Sea were systematically examined using microscopic and molecular tools. Similar to a previous histopathological study (Bythell *et al*., 2002), in the present study, BBD-affected coral tissues under the mat and at the boundary were characterized by pycnotic nuclei indicating necrotic or apoptotic processes. In the present study we showed histologically, for the first time, the actual penetration of the cyanobacteria filaments into the mesoglea of intact tissue in the region adjacent to the mat. Samples of the surface mucus layer taken from the coral tissue adjacent to the black band (INT) and assessed molecularly validate the presence of the cyanobacteria within this region. These sequences were identical to those obtained from the band (S) itself (see Fig. 4). The results strengthen the importance of the role of this group in BBD etiology. Similar to what was previously reported in bacterial bleaching in corals (see Banin *et al*., 2000) it is likely that one of the first steps of the infection process of BBD entails the penetration of microorganisms into the tissue. It is possible that the cyanobacteria facilitate this progression by secreting enzymes and toxins. Marine cyanobacteria are known to produce a variety of toxins including microcystin (Izaguirre *et al*., 2006). These toxins are known to cause cell mortality possibly via the apoptotic pathways (Botha *et al*., 2004; Teneva *et al*., 2005).

Cyanobacterial sequences obtained from the affected corals in the present study formed a cluster (similarity values were over 99%) with sequences previously identified from corals affected with BBD and RBD from the Indo-Pacific region (Frias-Lopez *et al*., 2004; Sussman

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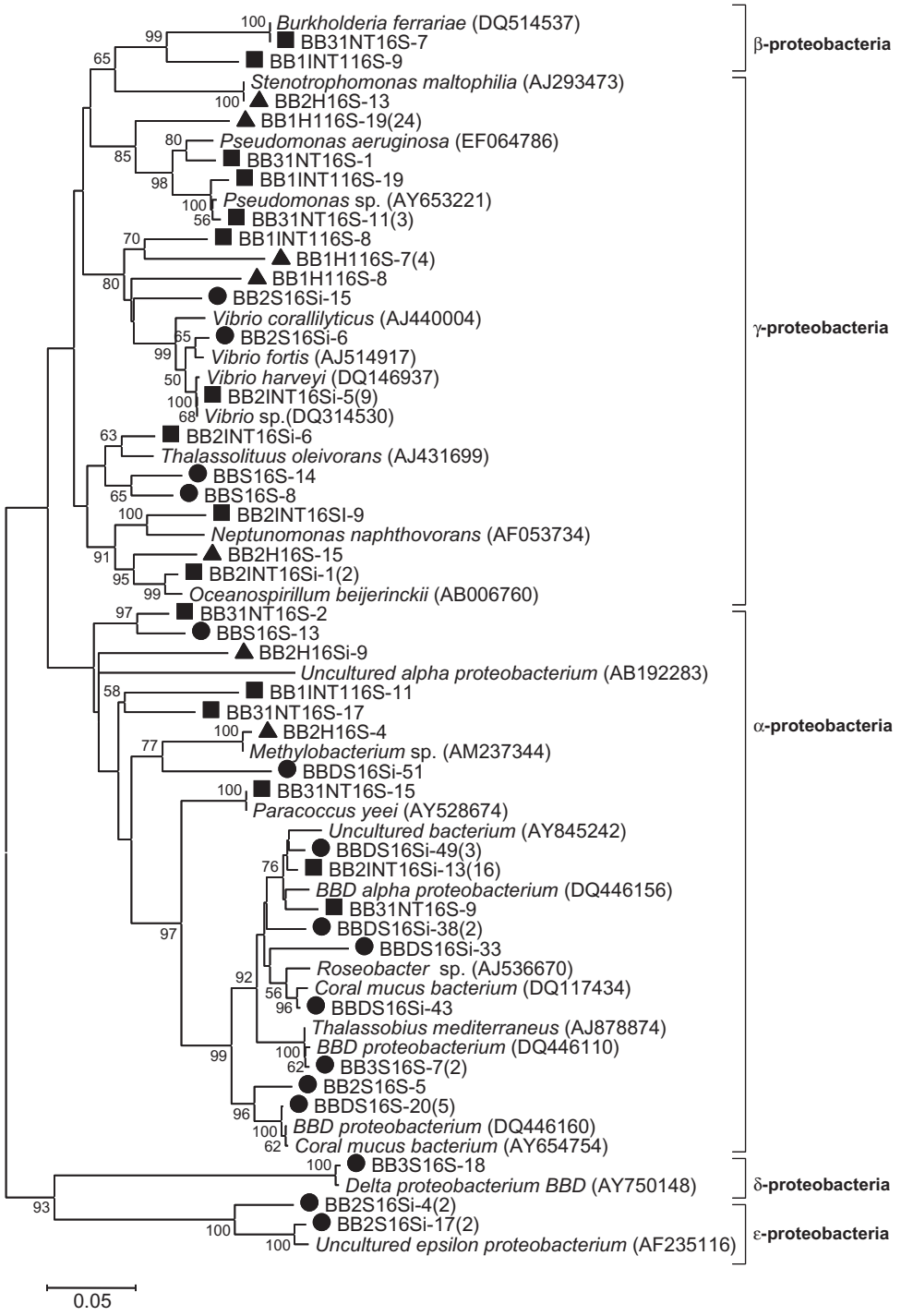

Fig. 5. Phylogenetic tree based on 16S rRNA gene of proteobacteria sequences that were retrieved from BBD corals. Geometric forms indicate the origin of the sequence according to the sampled area: (●, S) the black band, (■, INT) the surface mucus layer adjacent to the band, (▲, H) the surface mucus layer of apparently healthy coral tissue. Sequences that were retrieved using the modified 16S primers are indicated by '16Si'. The tree was constructed by the Neighbour-Joining method (Saito and Nei, 1987) with the Mega package (Kumar *et al*., 2004) using partial sequences of 16S rRNA gene. The numbers in parentheses indicate the total number of similar clones on the basis of ≥ 97% identity for each representative sequence. The bar represents five substitutions per 100 nucleotide positions. Bootstrap values (Felsenstein, 1985) are indicated at branch nodes.

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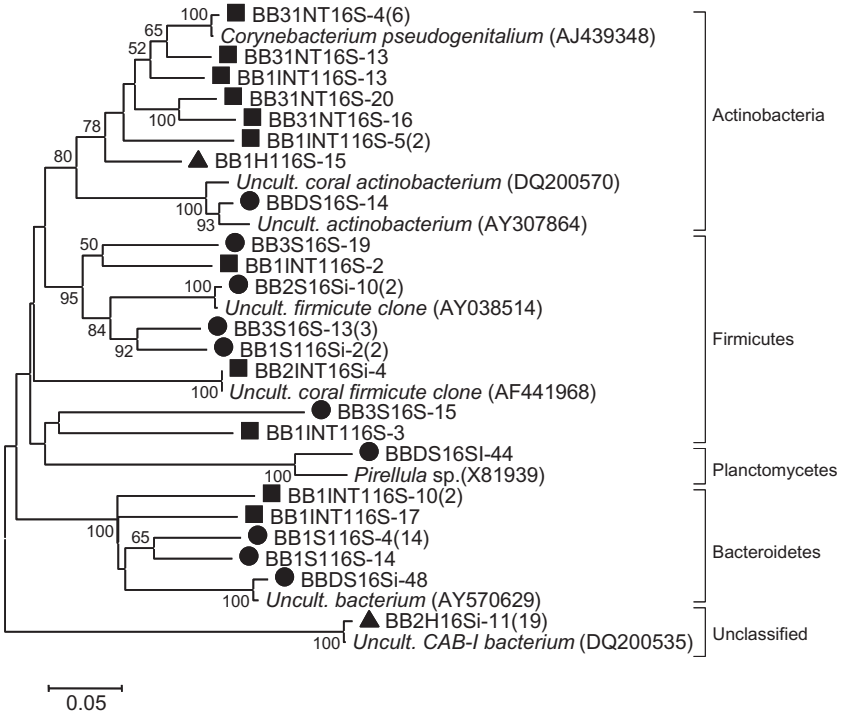

Fig. 6. Phylogenetic tree based on 16S rRNA gene of non-proteobacteria sequences that were retrieved from BBD corals. Geometric forms indicate the origin of the sequence according to the sampled area: (●, S) the black band, (■, INT) the surface mucus layer adjacent to the band, (▲, H) the surface mucus layer of apparently healthy coral tissue. Sequences that were retrieved using the modified 16S primers are indicated by '16Si'. The tree was constructed by the Neighbour-Joining method (Saito and Nei, 1987) with the Mega package (Kumar *et al*., 2004) using partial sequences of 16S rRNA gene. The numbers in parentheses indicate the total number of similar clones on the basis of ≥ 97% identity for each representative sequence. The bar represents five substitutions per 100 nucleotide positions. Bootstrap values (Felsenstein, 1985) are indicated at branch nodes.

et al., 2006). Interestingly, the similarity values to sequences obtained from the Caribbean region were high as well and ranged between 98% and 99% (Frias-Lopez *et al*., 2002, 2004). These results further strengthen the universal presence of certain cyanobacteria associated with BBD. A single cyanobacterial sequence was retrieved from the clone library BB2H, which represents the healthy part of a sick coral. The latter was 91% similar to *Prochlorothrix* genus, which was previously detected in the marine environment and in association with healthy *Montastrea franksii* colonies (Rohwer *et al*., 2001, 2002). This may demonstrate the presence of different cyanobacterial assemblages associated with healthy tissue from affected corals.

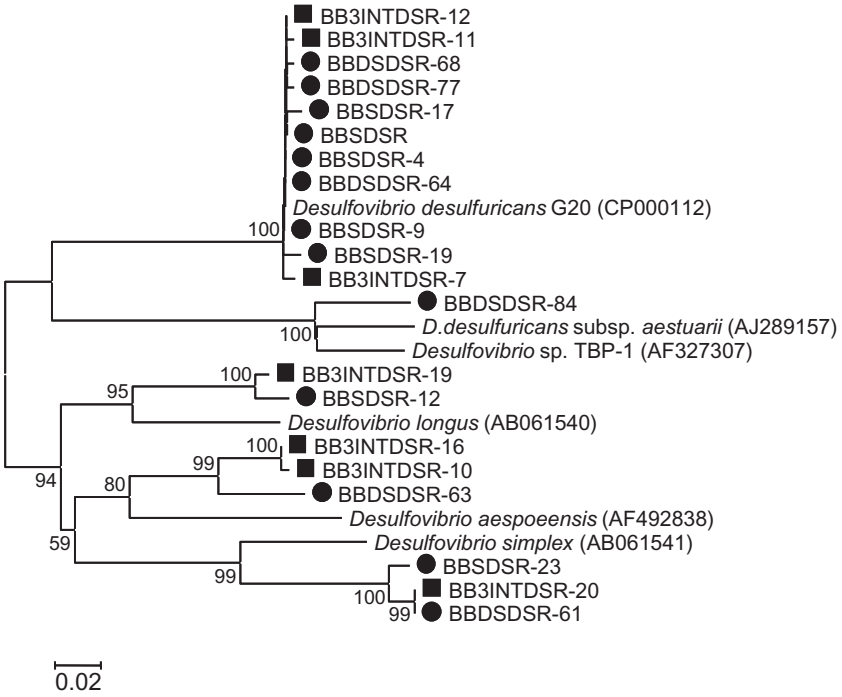

Fig. 7. Neighbour-Joining phylogenetic tree for the dissimilatory sulfite reductase gene *dsrA* of SRB showing relationship between sequences obtained from BBD mat in Eilat (BB, BBD and BB3 samples, see Table 1) and the closely related sequences of SRBs obtained from the GenBank. Geometric forms indicate the origin of the sequence according to the sampled area: (●, S) the black band, (■, INT) the surface mucus layer adjacent to the band, (▲, H) the surface mucus layer of apparently healthy coral tissue. Sequences that were retrieved using the modified 16S primers are indicated by '16Si'. The tree was constructed by the Neighbour-Joining method (Saito and Nei, 1987) with the Mega package (Kumar *et al*., 2004) using partial sequences of 16S rRNA gene. The bar represents two substitutions per 100 nucleotide positions. Bootstrap values (Felsenstein, 1985) are indicated at branch nodes.

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In the present study comparison of microbial communities was carried out within different areas of diseased colonies. Results showed that similar to previous studies on BBD (see Cooney *et al*., 2002; Frias-Lopez *et al*., 2002: Sekar *et al*., 2006) a high microbial diversity was revealed in the affected tissue (INT and S) in comparison with the healthy (H) area of the same colony (Fig. 3). A study by Pantos and colleagues (2003) on plague-like disease suggested that the microbial community of unaffected corals differ from those of the healthy area of affected corals. This phenomenon should be further investigated for BBD and other coral diseases.

Polymerase chain reaction-based techniques using traditional universal 16S rRNA gene primers are limited in the ability to reveal true diversity of microbial communities (Ben-Dov *et al*., 2006). This is particularly true when studying coral disease-associated microbial diversity. In a recent study, Pantos and Bythell (2006) stated that preferential carbon utilization methods suggest the presence of *Vibrio* species in white band disease-affected corals, yet this genus was not detected by molecular PCR-based techniques using traditional 16S primers. The authors (Pantos and Bythell, 2006) specifically bring up a possible limitation of the 16S primer set. The use of the modified primer set 8F-I/907R-I (where the 3′-terminus of both primers were substituted by inosine) has been proven useful as a tool to overcome the shortcomings of universal 16S rRNA gene primers (8F and 907R) (Ben-Dov *et al*., 2006). Using this technique we were able to retrieve sequences belonging to a number of genera, in particular sequences belonging to the genus *Vibrio*, a group that was under-represented in BBD clone libraries analysed to date. In the present study the vibrionic sequences retrieved from the S and INT libraries were closely related to known *Vibrio* species (Fig. 5). This genus includes species that were previously documented as pathogens of corals and other marine organisms (Kushmaro *et al*., 1996; Harvell *et al*., 1999; Ben-Haim *et al*., 2003). For example, *Vibrio* sequences found in the present study clustered together with the coral pathogen *Vibrio coralliilyticus* (Accession No. AJ440004), a bacterium known to cause rapid tissue lysis in the stony coral *Pocillopora damicornis* by metalloproteinase at elevated temperatures (Ben-Haim *et al*., 2003; Rosenberg and Falkovitz, 2004). An additional clone BB2INT16Si-7 (Accession No. EF089442) was similar (99%) to an algicide producing *Vibrio* sp. (Accession No. AB180389). A similar capability was reported for another *Vibrio* species, *Vibrio shiloi*. The latter, which is the causative agent of bacterial bleaching in the coral *Occulina patagonica*, is known to produce a proline-rich peptide that inhibits photosynthesis and a protease that lyses zooxanthellae (Ben-Haim *et al*., 1999; Banin *et al*., 2000; Rosenberg and Falkovitz, 2004). As in several cases, virulence of *Vibrio* is associated with protease activity (e.g. Hada *et al*., 1984; Ben-Haim *et al*., 1999; Banin *et al*., 2000; Ben-Haim and Rosenberg, 2004; Rosenberg and Falkovitz, 2004) it is possible that this group of organisms may play a key role in the etiology of BBD. The observed characteristic lysis of coral tissue in the disease interface may therefore be associated with proteolytic enzyme activity.

In our clone libraries, bacterial sequences were derived from a wide taxonomic range, with the majority of clones closely related to bacteria previously described from marine environments and some previously reported as general coral-associated bacteria. A distinct cluster of sequences originating form the surface mucus layer of the healthy coral region and obtained solely with the modified 16S primers showed high similarity (99%) to CAB-I bacteria (Accession No. DQ200535, DQ200526, DQ200473), which were associated with the shallow water colonies of the coral *Montastrea annularis* (Klaus *et al*., 2007). Among the sequences originating from the black band and the interface regions, several (BB2S16Si-10, BB2S16Si-12 Accession No. EF089454 and EF089455 respectively) showed high homology (99%) to uncultured *Firmicute* clones CD4C3 (Accession No. AY038514), CD12A1 (Accession No. AF441968) and clone 34-36 (Accession No. AF473969) previously detected from BBD-infected corals (Cooney *et al*., 2002; Frias-Lopez *et al*., 2002). Four sequences belonging to the clone library BB3INT16S were highly homologous (99%) to bacteria belonging to the genus *Corynebacterium* (Accession No. AY494655, AJ439348) known to contain pathogenic species.

Previous studies dealing with the BBD consortium have addressed the role of SRB along with the other key players (Cooney *et al*., 2002; Frias-Lopez *et al*., 2002; 2004; Sekar *et al*., 2006; Viehman *et al*., 2006). Bacteria belonging to the genus *Desulfovibrio* have been identified in the BBD mat and on denuded coral skeleton behind the band in corals from different geographic locations (Cooney *et al*., 2002; Frias-Lopez *et al*., 2002; 2004; Viehman *et al*., 2006). A recent study by Viehman and colleagues (2006) focused on cultured *Desulfovibrio* spp. from BBD corals in the Dominican and Florida Keys reefs. In that study, 16S rRNA gene sequences of SRBs selectively cultured from six BBD bands on four coral species were compared. *Desulfovibrio* bacteria are known to reduce sulfate to sulfide and are known to be associated with disease processes (Pitcher and Cummings, 1996). Interestingly, in our library the one sequence (BB3S16S-18 Accession No. EF089473, Fig. 5) detected using 16S rRNA gene primer was similar by 98% to the cultured *Desulfovibrio* spp. (Accession No. AY750148 with high homology to *Desulfovibrio* sp. TBP-1, Fig. 7) isolated from BBD of *Siderastrea siderea* from the Caribbean (see Viehman *et al*., 2006). Eight out of 10 cultures sequenced by Viehman and colleagues (2006) were highly homolo-

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gous to a strain of *Desulfovibrio* which was originally isolated from marine sediment. The authors specifically mention that the use of 16S rRNA gene was 'imposed' on them by the lack of data available from other BBD studies and urged the use of functional or physiologically related genes in the study of SRBs (Viehman *et al*., 2006). Therefore, in order to investigate the diversity of SRB associated with BBD we used specific primers for the amplification of the dissimilatory sulfite reductase gene (*dsrA*). The sequences obtained in our study were closely related to four distinct species of *Desulfovibrio* out of which 46% showed high homology (98–99%) to *D. desulfuricans* G20 (Accession No. CP000112) (see Fig. 7). Moreover, the sequences which cluster with the latter belong to all three libraries, suggesting that this strain is present regularly in the BBD mat and adjacent to it and is neither season- nor species-specific. The similarity values of the other SRB sequences to known species of *Desulfovibrio* were lower and ranged from 83% to 92%. Surprisingly our use of specific primers for the amplification of the *dsrA* gene yielded only sequences that are closely related to *Desulfovibrio* species with no representatives of other genera of SRBs. This may imply the dominance of this genus in BBD-affected corals. BLAST searches performed with both *dsrA* primers that were used in our study revealed that both of them are capable of amplifying SRBs belonging to different families and even phyla, thus negating the possibility of primer-induced bias. Using the *dsrA* primers we were able to identify a *Desulfovibrio* clone (BBDSDSR-84 Accession No. EF089550) with a similarity value of 94% to *Desulfovibrio* sp. TBP-1 (Fig. 7), a fact that may suggest that this clone is related to the clone in our 16S library (Clone No. BB3S16S-18; Accession No. EF089473) (Fig. 5) and that may be related to the *Desulfovibrio* spp. (Accession No. AY750148) isolated by Viehman and colleagues (2006). As mentioned above, in the present study only one sequence of SRB was detected using the universal bacterial 16S rRNA gene primers. This fact further highlights the advantages of using a functional gene such as the *dsrA* as a tool in the study of SRB diversity (Wagner, 2005; Wagner *et al*., 1998). We suggest using additional functional genes such as adenosine-5′-phosphosulfate (APS) reductase (Rabus *et al*., 1999; Friedrich, 2002) in order to achieve better resolution in the identification of this group from diseased corals.

Similar to what was reported in human tissues (Pitcher and Cummings, 1996), experimental studies on corals showed that artificial exposure to conditions of anoxia and sulfide is lethal (Richardson *et al*., 1997; E. Kramarsky-Winter, pers. obs.). Black band mat was found to be rich in sulfide at times exceeding concentrations of 800 μM (Carlton and Richardson, 1995). Studies using microelectrodes showed that there are microenvironments within the black band and that the interface with the decaying coral tissue is anaerobic, containing variable levels of sulfide (Carlton and Richardson, 1995). It is thus likely that these sulfide levels affect coral tissue, at least at the interface between the band and healthy tissue (Carlton and Richardson, 1995). Interestingly Richardson and Kuta (2003) found that black band cyanobacteria, both in culture in the laboratory and freshly collected, were able to perform oxygenic photosynthesis in the presence of sulfide, an unusual capability for cyanobacteria (Stal, 1995) that may explain the presence of these organisms together with SRBs in the mat.

The complex nature of BBD as revealed by numerous morphological and molecular studies, and the lack of success in isolating a single causative agent, suggest that this disease can fall into the category of polymicrobial diseases (see Frias-Lopez *et al*., 2004). Diseases caused by combinations of microorganisms (e.g. viruses, bacteria, fungi and parasites), termed polymicrobial diseases, are being recognized with increasing frequency by the medical community (Brogden *et al*., 2005). In these infections, the presence of one microorganism generates a niche for other pathogenic microorganisms to colonize. One microorganism predisposes the host to colonization by other microorganisms, or two or more non-pathogenic microorganisms together cause disease. Interestingly, some polymicrobial diseases have been reported to include SRB that are believed to contribute to the disease process (Pitcher and Cummings, 1996; Edmond *et al*., 2003). For example, periodontal lesions are comprised of a complex microbiota that may exceed 500 different species and may include SRB (Moore and Moore, 1994; Langendijk-Genevaux *et al*., 2001). It is possible that similar processes exist in corals. The recognition of BBD as a polymicrobial disease requires further research in order to thoroughly characterize the key microbial players (e.g. vibrios, cyanobacteria and SRB) and to elucidate the complex interactions occurring between them. Current efforts therefore should be invested towards isolation of various potential pathogenic candidates and utilizing them singly and in combination to verify their pathogenicity by applying Koch's postulates.

Experimental procedures

Black band disease survey

A SCUBA survey of the affected corals was qualitatively carried out by recording affected species from the Inter-University Institute for Marine Science reef at Eilat, Israel, in the northern Red Sea (29°51′N, 34°94′E).

Sample collection

Infected corals *Favites* sp. and *Favia* sp. were detected in the reef across from the Inter-University Institute for Marine Science. Five samples of the diseased corals collected at a

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different season were analysed (see Table 1). Every coral was sampled at the black band region ('S'), three were also sampled at the interface ('INT') and two in the healthy ('H') regions. The black band region was collected using a needleless syringe. The interface region adjacent to the band and a region far from the band, where the tissue seemed healthy, were sampled using plastic bacteriological loops that were used to rub off the coral surface microlayer. The loops (three for each sample) were transferred to plastic 15 ml of sterile vials with minimal amount of seawater and sealed underwater. As previous studies demonstrated that cyanobacteria are abundant in the skeleton of massive corals (Fine and Loya, 2002), samples from these regions were collected using loops in order to minimize tissue damage and the sampling of cyanobacteria from the skeleton beneath the tissue. This method also enables re-sampling of the same coral. Samples were frozen immediately and transfer frozen to the lab. In addition, core samples of the BBD interface, and healthy tissues were collected for histology using a 2-cm-diameter aluminum corer. The cores were collected from two diseased colonies of *F. favus* fixed in 4% Formalin buffered in seawater. The cores were enrobed in agarose in order to maintain tissue integrity following decalcification, and decalcified using a 1:1 mixture of formic acid and sodium citrate. They were processed in a Citadel tissue processor and 6 μm sections were stained using standard haematoxylin/eosin and observed under the microscope.

DNA extraction

Frozen samples were thawed and centrifuged for 1 min at maximal speed in order to pellet the mucus/mat. Genomic DNA was extracted by PowerSoil purification kit (Mo Bio Laboratories, Solana Beach, CA, USA) according to the manufacturer's instructions.

Polymerase chain reaction amplification

Total DNA was amplified using Biometra TGradient thermocycler (Biometra GmbH, Göttingen, Germany) by PCR using specific 16S rRNA gene primers for bacteria: forward primer, 8F [5′-GGATCCAGACTTTGAT(C/T)(A/C)TGGCTCAG], taken and modified (8F primer was shortened from the 5′ end; Felske *et al*., 1997) and reverse primer, 907R [5′-CCGTCAATTCCTTT(A/G)AGTTT-3′; Lane *et al*., 1985]. In addition, modified 16S rRNA gene primers 8F-I and 907R-I were obtained by replacement of the last nucleotide at the 3′-terminus with inosine (Ben-Dov *et al*., 2006). In order to retrieve SRB sequences, specific primers DSR1F (5′-ACSCACTGGAAGCACG-3′) (Wagner *et al*., 1998) and DSR10R (5′-CGGTTYTTGCCYTCTTCC-3′) (modified from Friedrich, 2002) for the dissimilatory sulfite reductase gene *dsrA* were used. Polymerase chain reaction mixtures included a 12.5 μl ReddyMix (Master mix containing 1.5 mM $MgCl_2$ and 0.2 mM concentration of each deoxynucleoside triphosphate) (ABgene, Surrey, UK), a 1 pmol each of the forward and reverse primers, 1–2 μl of the sample preparation, plus water to bring the total volume to 25 μl. When amplification failed with ReddyMix, the FailSafe PCR System kit was used instead (Epicentre, Madison, WI). An initial denaturation-hot start of 4 min at 95°C was followed by 30 cycles of the following incubation pattern: 94°C for 40 s, 54°C (48°C for 8F-I/907R-I) for 40 s, and 72°C for 60–120 s. The procedure was completed with a final elongation step at 72°C for 20 min.

Clone library construction and sequencing

Polymerase chain reaction products were purified by electrophoresis through a 1% agarose gel, stained with ethidium bromide and visualized using a UV transilluminator. The desired bands were excised from the gel and the DNA was purified from the gel slices using the Wizard PCR Prep kit (Promega, Madison, WI). The gel-purified PCR products were cloned into the pCRII-TOPO-TA cloning vector as specified by Invitrogen (Carlsbad, CA) and transformed into calcium chloride-competent HD5α *Escherichia coli* cells according to manufacturers' instructions. Plasmid DNA was isolated from individual clones by Wizard Plus SV Minipreps DNA purification system (Promega, Madison, WI). Sequencing with 8F and DSR1F primers were performed by ABI PRISM dye terminator cycle sequencing ready reaction kit with AmpliTaq DNA polymerase FS and DNA sequencer ABI model 373A system (Perkin-Elmer). Each sequence was designated with a name containing the sample name (e.g. BB2), sample region (H, INT or S), primer set used for amplification (16S, 16Si, DSR) and a serial number.

Sequence analysis

Molecular Evolutionary Genetics Analysis (MEGA), version 3.1 (Kumar *et al*., 2004), was used to de-replicate the libraries of 16S rRNA gene sequences for subsequent analyses, by comparing all the sequences in a data set to each other, grouping similar sequences together by ≥ 97 similarity, and choosing a representative sequence from each group. All 16S rRNA gene sequences of each group were first compared with those in the GenBank database with the basic local alignment search tool BLAST (http://www.ncbi.nlm.nih.gov/blast/blast.cgi). Classifier program (version 1.0; assign 16S rRNA gene sequences to a taxonomical hierarchy) and Library Compare program (compare two sequence libraries using the RDP classifier) available at the Ribosomal Database Project-II web site (http://rdp.cme.msu.edu/, Maidak *et al*., 1997) were used to find diversity on different ranks of related sequences. To control for the occurrence of possibly chimeric sequences, all sequenced clones were analysed by the Chimera Check program of the RDP database (version 2.7; Maidak *et al*., 1999). The sequences from appropriate libraries were aligned using CLUSTALW with the MEGA package (Kumar *et al*., 2004) and positions not sequenced in all isolates or with alignment uncertainties were removed. Phylogenetic trees were constructed by the Neighbour-Joining method (Saito and Nei, 1987) using the Mega package (Kumar *et al*., 2004). Bootstrap re-sampling analysis (Felsenstein, 1985) for 100 replicates was performed to estimate the confidence of tree topologies. A Shannon–Weiner index for diversity was applied using the FASTGroupII program, with default of 80% similarity in sequence match (Yu *et al*., 2006; http://biome.sdsu.edu/fastgroup/fg_tools.htm).

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