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Caribbean Placozoan Phylogeography

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Abstract. We here address placozoan distribution and phylogeography in five locations in the Caribbean Sea. We performed a coarse-resolution presence/absence survey of placozoans in Belize, Bermuda, Grenada, Jamaica, and Panama and a fine-resolution study of the distribution of placozoans in Twin Cays, Belize. Placozoans were recovered in every country sampled. Animals were sequenced at the mitochondrial 16S rDNA locus, and our analysis identified four of the five previously identified clades present in the Caribbean. In addition, we discovered two new haplotypes within one of these clades, and we found sympatric clades in Belize, Bermuda, Jamaica, and Panama. These studies provide further molecular evidence for species diversity within the Phylum Placozoa.

Introduction

Since its discovery, *Trichoplax adhaerens* has been classified as the sole species within the phylum Placozoa (Grell, 1971a), purported to reproduce sexually (Grell, 1971b, 1972), and discovered across the world. Today we know that the Placozoa actually consists of several clades based on 16S rDNA, LSU, SSU, and ITS genotyping (Voigt *et al.,* 2004) and that sexual reproduction is indeed part of the life cycle in at least one of these clades (Signorovitch *et al.,* 2005). While significant progress continues to be made in the field of placozoan biology—as exemplified by the placozoan genome sequencing project presently underway—the phylogeography of this group has remained poorly understood.

The first placozoan record dates from 1883 when zoologist F. E. Schulze discovered the microscopic amoeba-like animals in an aquarium tank containing seawater from the Gulf of Trieste in the Mediterranean Sea. Schulze (1883) aptly named this animal *Trichoplax adhaerens* for its flattened and ciliated body that held fast to the aquarium wall. Because *T. adhaerens* lacked any definite outline or organs and possessed a uniquely simple yet highly integrated body plan, Schulze declared it the most primitive of all metazoans. Almost a century of stasis elapsed before further investigations on this simple animal resumed. The German protozoologist K. G. Grell (1971a) rediscovered *T. adhaerens* while examining an algal sample from the Red Sea near Eilat, Israel. By stably maintaining *T. adhaerens* on a diet of *Pyrenomonas salina* (Cryptophyceae), Grell and Benwitz (1971) observed in detail its histological organization. *T. adhaerens* lacks a basement membrane and has only four somatic cell types. Because of its unique body plan and histological organization, Grell established the phylum Placozoa (Grell, 1971a) to house this singular species.

Since the resurgence of interest in placozoan biology in the 1970s, these animals have been discovered in numerous marine aquaria (Miller, 1971; Ivanov *et al.,* 1980; Martinelli and Spring, 2003; Voigt *et al.,* 2004) and seawater systems (Pearse, 1989; Maruyama, 2004), and in the wild where they have been sampled from tropical and subtropical latitudes (Maruyama, 2004; Voigt *et al.,* 2004). In 1980, Ivanov and colleagues discovered placozoans in a marine aquarium in the Moscow Zoo, Russia (Ivanov *et al.,* 1980). They noted slight morphological differences in the so-called "brown bodies" between the Moscow and Red Sea isolates (referenced in Aleoshin *et al.,* 2004), and for this reason, the Moscow isolate was named *Trichoplax* sp. instead of *Trichoplax adhaerens.* In 1987, Grell and Lopéz-Ochoterena (p. 255) discovered placozoans in what they referred to as "a small underground seawater deposit" near a dock in Quintana Roo, Mexico. Although the authors were unable to culture these placozoans with the usual *Pyrenomonas* food

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source, they were successful with the green alga *Chlorella.* This dietary preference led them to hypothesize that the animals sampled in Mexico belonged to a different group of placozoans from the established Red Sea strain.

In 1989, V. B. Pearse reported on the first record of placozoans in Honolulu, Hawaii. These animals were discovered on glass microscope slides that had been submersed in running seawater tanks for 2 to 3 weeks. A thin film of bacteria, algae, or both, and an assemblage of microorganisms such as foraminiferans, sponges, hydroids, ascidians, copepods, and nematodes characterized these slides. Pearse suggested that these placozoans most likely belonged to the only described species, *T. adhaerens,* because the morphological features and the growth rate of the placozoans found in Hawaii were consistent with previous observations by Grell (1971a) and measurements reported by Ruthmann (1977).

Placozoans have also been documented at widely distributed sites in the Caribbean and throughout the tropical Pacific. The most thorough field study of placozoans to date is a survey on the north coast of Papua New Guinea (V. B. Pearse, UC Santa Cruz, pers. comm., 2005). The survey aimed specifically at understanding the distribution and abundance of placozoans in a variety of environments ranging from a laboratory seawater system, to rubble, mangroves, and reefs. Glass microscope slides used as settling plates were either attached to solid substrata or suspended in the water column for 2 to 3 weeks before retrieval and screening. Placozoans were found to be widely distributed among these sampling locations, with the exception of sandy or rubble substrates, which were devoid of placozoans. As in the Hawaiian study, placozoans were found associated with the now familiar thin film of bacteria, algal crusts and filaments, ciliates, polychaete worms, calcareous sponges, and hydroids. Like Grell and Lopez-Ochoterena (1987), Pearse also found survival and growth variability among the New Guinea isolates.

The longest field study of placozoans was based in Shirahama, Japan, from 1989 through 2000 (Maruyama, 2004). Placozoans were sampled monthly, using glass microscope slides, from sites about 1 m below the mean tidal level. Natural substrata were also sampled, such as stones, shells, and hard corals. A running seawater tank system containing hard corals was also set up in the laboratory to sample for placozoans. The 11 years of continuous sampling show that placozoans are present year-round on both the slides and substrate material at the field site and on the laboratory slides in the running seawater system. Variation in the abundance of placozoans sampled within each year was reflected in a sharp increase during the late summer and early winter.

In a seminal paper by Voigt *et al.* (2004), DNA sequences from placozoans collected around the world were compared at four loci (SSU, LSU, ITS, and 16S rDNA) to construct a phylogeny of this group. From these studies, the investigators concluded that the Placozoa is composed of at least five highly divergent clades. Thus, although the past records on the occurrence and distribution of placozoans do contribute significantly to our knowledge of the ecology of placozoans, the genetic information necessary to understand the patterns in biogeography of these clades has only just begun to surface from molecular diversity analyses.

The work described here aimed at understanding placozoan phylogeography with a focus on the Caribbean Sea. Specifically, we report on a molecular survey of partial 16S ribosomal DNA sequences from 64 samples encompassing five Caribbean nations and on a detailed analysis of placozoans found in the small mangrove island of Twin Cays, Belize, where we provide the first high-resolution phylogeographic study of the Placozoa.

Materials and Methods

We undertook both a coarse-resolution study, designed to sample for the presence and diversity of placozoans in Belize, Bermuda, Grenada, Jamaica, and Panama, and a fine-resolution study, designed to sample for the presence and diversity as well as the distribution and abundance of placozoans in Twin Cays, Belize. All animals were collected using glass slides encased in modified plastic microscope slide boxes, as designed by Pearse (UC Santa Cruz, pers. comm., 2005). We cut the top and bottom panels from slide boxes (9.5 cm tall by 8.2 cm wide), leaving a narrow 1-cm border. Five glass slides (7.5 cm by 2.5 cm) were evenly spaced inside, and the box tops and bottoms were secured together by cable ties. Either cable ties or rope were used to attach the slide boxes to mangrove roots or dock piles at a depth of 40 cm or greater, and the boxes were exposed to the marine environment for 2 to 7 weeks. Each slide box was recovered, placed separately in its own disposable plastic bin while still submerged, and returned to the laboratory for immediate processing. Each slide was then placed into a large petri dish (14 cm diameter by 2 cm high) fitted with two parallel runners to prevent slides from touching the bottom of the dish. Both sides of each slide were screened for placozoans by using a Zeiss Stemi SR or Wild dissecting microscope. After all slides from a single box were screened, the petri dish was thoroughly rinsed with distilled water before the next box of slides was screened.

In our Caribbean-wide coarse sampling, we deployed groups of slide boxes largely in mangrove habitats and boat docks. The deployment sites were chosen for their proximity to marine field stations, as easy access to laboratory facilities was necessary for conducting the animal screens. Animals were collected near or at the Smithsonian Institution Marine Station at Carrie Bow Cay in Belize, the Bermuda Biological Station for Research (BBSR), St. George's University Marine Laboratory in Grenada, Discovery Bay

Marine Laboratory in Jamaica, and the Smithsonian Tropical Research Institute (STRI) in Bocas del Toro in Panama. For details of the deployment sites, habitats, and number of boxes deployed, see Table 1.

Slides positive for placozoans were individually placed in 50-ml Falcon tubes completely filled with seawater and transported within 2 days to the laboratory at Yale University. Animals collected from Twin Cays, Belize, during the summer of 2003 were first cultured at the marine station in Carrie Bow Cay for 3 weeks, then transported as above to Yale. In the laboratory, single isolates from each slide were cultured in glass petri dishes filled with about 250 ml of filter-sterilized artificial seawater (Reef Crystals, Marineland Labs, Moorpark, CA), salinity 37 psu, supplemented with 250 μl of Micro Algae Grow (Florida Aqua Farms, Dade City, FL) and 2 ml of a stationary phase culture of *Pyrenomonas salina.* Genomic DNA was extracted from successfully cultured isolates following published protocols (Signorovitch *et al.,* 2005).

Since our second goal was to obtain a fine-resolution phylogeographic map of Twin Cays, Belize, we systematically sampled the margins of this island over the course of two summer field seasons (August 2003 and June 2004). We chose Twin Cays because of its small size; proximity to Carrie Bow Cay, the Smithsonian Marine Station in Belize; and prior knowledge that placozoans occur in this region. We sampled at intervals of at least 10 m for a total of 150 slide boxes. Although no particular habitat in Twin Cays was purposely selected for sampling, mangrove trees line most of the island, and therefore, most of our sampling sites reflected the fauna and flora associated with a mangrove ecosystem. One exception was a sandy beach habitat in the northwest part of the island.

For each single animal isolate, a region of the mitochondrial 16S ribosomal DNA (16S rDNA) was amplified by polymerase chain reaction, using the forward 5′-CGAGAAGACCCCATTGAGCTTTACTA-3′ and reverse 5′-TACGCTGTTATCCCCATGGTAACTTT-3′ primer pair under the following PCR conditions: 95 °C denaturation for 2 min; 5 cycles: 95 °C for 30 s, 63 °C for 30 s, and 72 °C for 1 min; 5 cycles: 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min; 20 cycles: 95 °C for 30 s, 61 °C for 30 s, and 72 °C for 1 min; 72 °C final extension for 10 min. The amplification products were purified using QIAquick (Qiagen) and sequenced in both directions with PCR primers by either dGTP BigDye or TaqFS dye terminator cycle sequencing reactions using ABI PRISM 3730 DNA sequencers (Applied Biosystems, Inc.) at the W. M. Keck Biotechnology Center at Yale University. ABI chromatogram files and DNA sequences were analyzed by the LaserGene software package, Ver. 6 (DNASTAR, Inc.) and aligned by CLUSTALW (Chenna *et al.,* 2003) with adjustments performed manually. DNA sequences were deposited in the GenBank database, searchable by strain name or accession numbers: DQ389756–DQ389767 and DQ389769–DQ389885. Placozoan samples were also deposited in the Yale University Peabody Museum and are identified by strain name under accession number 10653. Maximum parsimony (MP) phylogenetic reconstruction was performed only on unique haplotypes in addition to those available from GenBank (accession numbers: AY652522–AY652529 and AY603696) using the heuristic search option in PAUP* 4.0b10 (Swofford, 1998) with default values. Bootstrap values for the MP tree were obtained by running 10,000 bootstrap replicates under the full heuristic search method. Bayesian likelihood inference was carried out using the K80+G model of evolution obtained from Modeltest, ver. 3.7 (Posada and Crandall, 1998). Bayesian posterior probabilities were obtained from the software MrBayes, ver. 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) using Nchains = 4, temp = 0.5, and running 1,000,000 Markov Chain Monte Carlo generations, sampling at every 100 generations with a burn in of 25% (potential scale reduction factor $\approx$ 1.0 for all parameters).

Heretofore, the nomenclature used to describe known placozoan haplotypes was that introduced by Voigt *et al.* (2004). We here build upon this naming convention by introducing two additional terms. First, we classified the haplotypes identified by Voigt *et al.* (2004) into five monophyletic clades: Clade I contains haplotypes H1 and H2; Clade II contains haplotype H3; Clade III is composed of haplotypes H6, H7, and H8; Clade IV contains haplotype H5; and finally, Clade V is represented by haplotype H4. For any new haplotype discovered in this study, we identified its closest known haplotype by sequence homology and named it in a fashion consistent with the already established haplotype nomenclature. For example, a new haplotype highly similar to H4 would be named H4-2 and classified as a member of Clade V. The second term we introduce here is a nomenclature for the individual strains used in this study. All individuals were given a name that begins with their two-letter country code of origin, followed by a laboratory strain identifier. For instance, individuals BZ10101, JM511, and GD711e originate from Belize, Jamaica, and Grenada, respectively.

Results

The results of our sampling efforts are summarized in Table 1. Placozoans were found in all five countries and in the majority of habitat types sampled, but not at every deployment site. Mangrove habitats consistently yielded placozoans regardless of whether the roots possessed a rich epifauna. For example, placozoans were collected from mangrove roots in Mangrove Bay and Walsingham Bay, Bermuda, where the roots were covered with epiphytes but had few epifauna. Placozoans were even found in the least pristine habitats sampled, including the muddy Mangrove

Table 1

Summary of sampling locations and haplotypes discovered

Country Deployment site	Habitat type	Deployment date (month, year)	Exposure time (weeks)	No. boxes deployed	No. boxes positive	No. clones genotyped	Clade: haplotype (individual strain names)
Belize							
Carrie Bow Cay	boat dock	08/2002	2	12	8	0	
Carrie Bow Cay	seawater system tank	08/2002	2	1	0	0	
Culew Cay	reef	08/2002	2	2	2	0	
Twin Cays	mangrove roots	08/2002	2	9	4	7	I: H2 (BZ514, BZ516, BZ534, BZ611, BZ613) III: H8 (BZ10101) V: H4 (BZ931)
Twin Cays	mangrove roots	08/2003	3	71	29	12	II: H3 (BZ12) III: H6 (BZ227) III: H7 (BZ45, BZ312, BZ651, BZ672) III: H8 (BZ264, BZ384, BZ46, BZ413) V: H4 (BZ42, BZ49)
Twin Cays	sandy beach	06/2004	3	5	0	0	
Twin Cays	mangrove roots	06/2004	3	74	35	20	II: H3 (BZ2423, BZC12, BZD10, BZD12) III: H6 (BZD5, BZD11) III: H7 (BZB8, BZE12, BZF2, BZF4) III: H8 (BZC2, BZC6, BZE8, BZF1) V: H4 (BZB11, BZD3, BZD8, BZE9, BZF3, BZF5)
Bermuda		08/2005	3				
Coot Pond	shallow open pond lined with *Zostera*			5	5	4	V: H4-2 (BMCP24, BMCP41, BMCP51) V: H4-3 (BMCP34)
Private dock in St. David's	boat dock			2	1	1	V: H4-3 (BMJP11)
Mangrove Bay	mangrove roots			5	4	3	V: H4 (BMMB11) V: H4-2 (BMMB42) V: H4-3 (BMMB33)
BBSR Concrete Beach	concrete boat ramp			2	1	0	
Mangrove Lake	murky enclosed tidal pond			5	1	0	
Walsingham Pond	enclosed tidal pond			5	4	4	V: H4-2 (BMWP53) V: H4-3 (BMWP12, BMWP22, BMWP45)
Walsingham Bay	mangrove roots			5	4	3	I: H2 (BMWB11) V: H4-2 (BMWB33) V: H4-3 (BMWB22)
	boat dock			2	0	0	
Grenada		03/2003	4				
Grand Anse	buoys in channel of sandy beach			4	2	0	
Grand Mal	fish and boat dock			4	4	1	III: H6 (GD711g)
Westerhall Point	mangrove roots			3	1	0	
Ft. Jeudy	mangrove roots			3	0	0	
Hog Island	mangrove roots			3	3	1	III: H6 (GD1721e)
Jamaica		02/2003	3				
Discovery Bay Mar. Lab.	mangrove roots			2	2	0	
	buoy over sandy bottom			2	1	0	
	fish dock			2	2	3	I: H1 (JM511) III: H8 (JM532, JM545)
	back reef			2	0	0	
	Thallasia bed			2	1	0	
Panama		06/2002	7				
STRI, Bocas del Toro	mangrove roots			2	1	5	I: H2 (PNa1, PNb4, PNb5, PNb6) III: H8 (PNa2)
	Thallassia bed			2	0	0	

Lake, also in Bermuda, which emitted foul odors reminiscent of sewage, and a "fish dock" at Grand Mal, Grenada, where the water was polluted with a layer of oil. Placozoans were not found near the sandy beach habitat in Twin Cays, but one sample was obtained from a buoy near the extensive sandy beach of Grand Anse, Grenada, and another from a buoy over sandy bottom in Jamaica.

Slides at the time of sampling typically displayed an early successional marine community comprising a biofilm of bacteria and algae, as well as a rich assembly of epifauna, including juvenile sponges, foraminiferans, tubeworms, hydrozoans, copepods, snails, bryozoans, and ascidians. Placozoans were often observed crawling over the biofilm and on hydrozoan stolons. Their colors varied from almost colorless to bright pink, depending on the substrate composition and color. Placozoans varied in size from less than 0.5 mm to as large as 3 mm in diameter, and most were found in an actively feeding state, as indicated by their flattened, stationary configuration. Slides on which placozoans were found typically harbored several individuals.

The results of our fine-scale sampling in Twin Cays, Belize, are mapped in Figure 1: colors indicate whether placozoans were absent or present at each site. Placozoans were distributed throughout this mangrove island, with the exception of the sandy beach habitat in the northwest region. Of the 159 slide boxes deployed around the perimeter of Twin Cays during the years 2002 through 2004, 68 were positive for placozoans. Most mangrove roots to which slide boxes were attached contained a variety of sponges, colonial ascidians, anemones, and filamentous algae. Turbidity in the water was low, and light intensity varied from high to low throughout the day. Slides displayed an assemblage of organisms similar to that seen in the coarse-resolution study, with the addition of the benthic ctenophore *Vallicula multiformis,* which was abundant on many of the recovered slides.

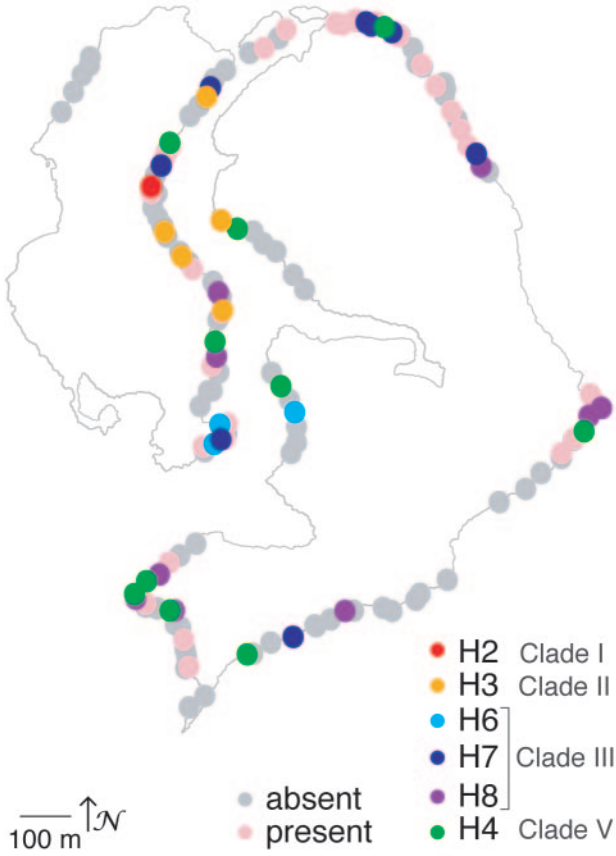

Figure 1. Map of Twin Cays, Belize. Dots on the map represent the sampling sites. Grey dots indicate sites from which placozoans were not detected. Pink dots represent sites from which placozoans were detected but not genotyped. The remaining colored dots represent sites positive for placozoans from which individual strains were genotyped. The habitat type at the five sampling sites in the northwestern-most part of the map is sandy beach; all other sampling sites are mangrove roots. (Map modified from a figure prepared by Molly K. Ryan for the Caribbean Coral Reef Ecosystems Program; used with permission.)

Using the combined coarse- and fine-resolution 16S rDNA data from this study and the 16S rDNA sequences, available from GenBank, of Voigt *et al.* (2004) (AY652522–AY652529, corresponding to haplotypes H1–H8, respectively) and Aleoshin *et al.* (2004) (AY603696), we constructed a partial 16S rDNA phylogeny of the Placozoa (Fig. 2). We were unable to sequence one region within this locus in part of our samples because of a guanine/cytoseine-rich hairpin secondary structure; therefore, the data have been partitioned into separate 5′ and 3′ regions. We used 73 DNA samples with an average concatenated length of 358 nt and a range from 292 to 406 nt. Due to large insertions or deletions (indels), the aligned data set spanned 480 nt. Removing indel sites reduced the data to 241 nt. Both parsimony (MP) and likelihood (Bayesian inference) methods returned the same tree topology for the well-supported nodes. On the basis of sequence divergence, the bootstrap percentages and Bayesian posterior probabilities, five clades emerged from the combined 73 samples, hereafter referred to as Clade I for haplotypes H1 and H2; Clade II for haplotype H3; Clade III for haplotypes H6, H7, and H8; Clade IV for haplotype H5; and Clade V for haplotypes H4, H4-2, and H4-3 (Fig. 2). Our coarse-and fine-resolution studies revealed seven of the eight haplotypes already described by Voigt *et al.* (2004), and in addition, two new haplotypes, H4-2 and H4-3, were discovered in Bermuda. Haplotype H4-2 differed from the H4 haplotype discovered by Voigt *et al.* (2004) by only two nucleotide substitutions, while haplotype H4-3 differed from H4 by four nucleotide substitutions. The last column of Table 1 lists the haplotypes detected during this study. In Bermuda ($n = 15$), 14 samples were found to belong to Clade V, while only one belonged to Clade I. Jamaican samples ($n = 3$) separated into Clades I and III. Grenadian samples ($n = 2$) belonged to Clade III,

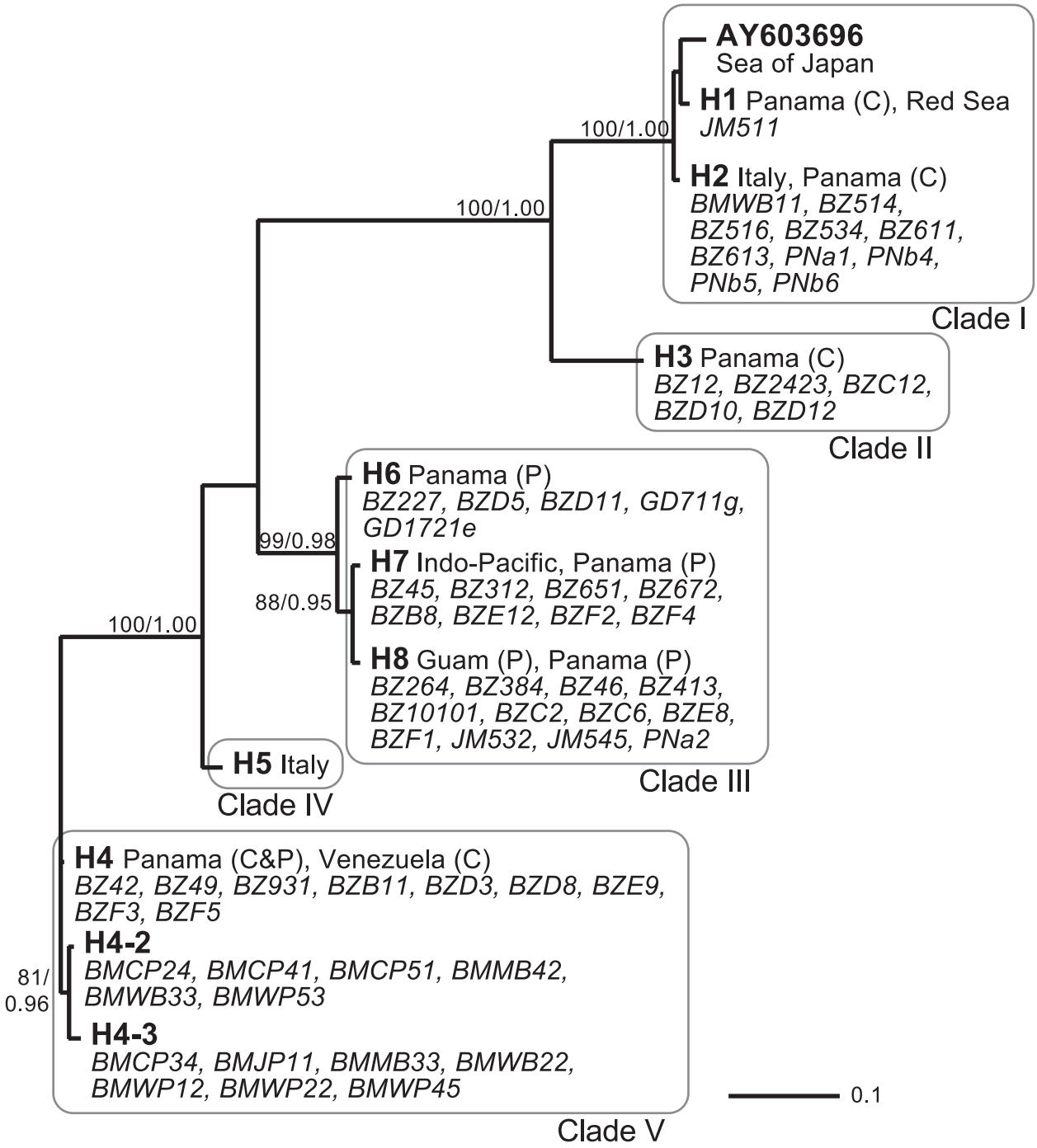

Figure 2. 16S rDNA phylogeny of combined placozoan samples from this study and sequences available in GenBank (represented by H1, H2, H3, H4, H5, H6, H7, H8, and AY603896). Haplotypes are represented in boldface type. Haplotypes H4-2 and H4-3 were newly discovered in this study. Individual strains sequenced in this study are italicized and labeled by their two-letter country code of origin (BM = Bermuda, BZ = Belize, GD = Grenada, JM = Jamaica, PN = Panama), followed by laboratory strain identifiers. Localities (countries and seas) sampled by Voigt *et al.* (2004) and Aleoshin *et al.* (2004) are explicitly listed next to haplotypes in bold. (C) = Caribbean Ocean, (P) = Pacific Ocean. Numbers at each internal node represent % bootstrap under maximum parsimony and Bayesian posterior probabilities, respectively (only values above 70% and 0.70 are shown).

and Panamanian samples ($n = 5$) fell into Clades I and III. In Twin Cays ($n = 39$) we uncovered six haplotypes that fall into Clades I–III and V. Figure 1 shows this haplotype distribution among the sampled sites in Twin Cays. The haplotype groupings (H2), (H3), (H6, H7, H8), (H4) correspond to Clades I–II and V, respectively. The three most abundant haplotypes were H7, H8, and H4, and the most diversity was observed in the main channel region that separates the two land masses composing the island.

Because only a subset of sampled animals were successfully established in laboratory culture during the 2003 Twin Cays field season (41%, see Table 1) and because sequence data were generated only for those cultured isolates, it is possible that the molecular data might be biased by differential viability of different clades. To address this possible survival bias in our 2003 data, in 2004 we used a CloneSaver FTA card (Whatman) to sample 19 placozoan individuals immediately upon their retrieval from the field, thus avoiding an intervening step of laboratory culture (for protocol, see Signorovitch *et al.,* 2005). Sequencing of these 19 DNA samples yielded only representatives of the known clades.

Discussion

The finding by Voigt *et al.* (2004) that the Placozoa is not a monotypic phylum raises a number of immediate questions that our study was designed to address. For instance, will extensive sampling result in the discovery of additional placozoan clades? Do the known clades display obvious geographic limits or habitat preferences? And finally, are these clades sympatric on an ecologically relevant spatial scale?

Our coarse-scale Caribbean-wide sampling revealed four of the five clades of placozoans previously identified (Voigt *et al.,* 2004). Clades I, III, and V all were widely distributed within the Caribbean basin. Voigt *et al.* had found Clade I to contain animals from the Red Sea, Italy (Mediterranean), and Panama (Caribbean side), and Aleoshin *et al.* (2004) identified a sample from the Sea of Japan (Fig. 2). Our study confirmed the presence of Panamanian animals in this clade and further added Belizean, Bermudian, and Jamaican representatives to it. We have also added Belizean animals to Clade II, which previously contained only a single Caribbean sample from Panama. Members of Clade III were previously identified only from the Pacific in Panama, the Indo-Pacific, and Guam (Voigt *et al.,* 2004), but we found that this clade displayed the most geographic diversity among the four clades. Indeed, Clade III contained animals from all but one of our sampling locations in the Caribbean and was widely distributed throughout Twin Cays, Belize. Finally, Clade V was represented by Panamanian (both Caribbean and Pacific sides) and Venezuelan samples (Voigt *et al.,* 2004), and our study has now added Belizean and Bermudian placozoans to this group. Because of difficulties in establishing animals in laboratory culture, our sampling efforts in different locales were highly uneven, preventing us from assessing geographic population structure across islands. Nevertheless, the fact that our Caribbean-focused study found identical haplotypes (H2, H3, H4, H6, H7, and H8) to those discovered in the worldwide sampling of Voigt *et al.* (2004) adds further support to the idea that placozoans, like other microbial eukaryotes (Finlay, 2002), are geographically unrestricted because of their small body size, pelagic stage in their life cycle, and large population size.

In the course of these studies, samplings were concentrated around docks, mangrove roots, and to a lesser extent, patch reefs. These habitat types were similar to those previously sampled (Voigt *et al.,* 2004). Inspection of Table 1 reveals no clear restriction of placozoans to any one of these habitats, with the exception of the sandy beach. The absence of placozoans in the sandy beach habitat of Twin Cays agrees with Pearse's observations in Papua New Guinea. The glass slides recovered from the sandy beach habitat in Twin Cays were very clean—almost devoid of any epifauna or flora. Because placozoans are often found on glass slides covered with a noticeable film of bacteria and algae as well a rich assemblage of other microorganisms, it is possible that such a diversity of organisms is necessary for their recruitment. The fact that we found several known haplotypes throughout the Caribbean and discovered only two new haplotypes that fall within an already reported clade suggests that most of the placozoan diversity in these habitats is adequately characterized in this region. It bears emphasis, however, that the epifauna of pilings and mangroves is only a tiny fraction of the possible habitats for placozoans, and further discoveries of placozoan diversity might take place in other habitats or in other parts of the world.

The lack of any obvious clade-specific habitat preference in our coarse-scale analysis is consistent with the results of our fine-scale study in the mangrove community of Twin Cays (Fig. 1). All four clades were isolated from this 92-hectare island. Members of Clades III and V were widespread throughout the island, whereas members of Clades I and II were found only within the Main Channel region dividing the two landmasses composing the island (Fig. 1). Clades III and V were the most abundant and widely distributed clades, while Clade I was especially rare. These results clearly indicate that the four clades occur in sympatry in Twin Cays on an ecologically relevant spatial scale. Furthermore, sympatry is likely to be a widespread phenomenon, as indicated by observations of multiple clades in Panama and Italy (Voigt *et al.,* 2004) and the fact that even a single slide from our Panamanian collection yielded representatives of two different clades (Table 1). The sympatry

of clades raises questions about the ecological dynamics among these grossly similar placozoans.

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