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REPORT

Genetic structure of the Caribbean giant barrel sponge *Xestospongia muta* using the I3-M11 partition of COI

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Abstract In recent years, reports of sponge bleaching, disease, and subsequent mortality have increased alarmingly. Population recovery may depend strongly on colonization capabilities of the affected species. The giant barrel sponge *Xestospongia muta* is a dominant reef constituent in the Caribbean. However, little is known about its population structure and gene flow. The 5′-end fragment of the mitochondrial gene cytochrome oxidase subunit I is often used to address these kinds of questions, but it presents very low intraspecific nucleotide variability in sponges. In this study, the usefulness of the I3-M11 partition of COI to determine the genetic structure of *X. muta* was tested for seven populations from Florida, the Bahamas and Belize. A total of 116 sequences of 544 bp were obtained for the I3-M11 partition corresponding to four haplotypes. In order to make a comparison with the 5′-end partition, 10 sequences per haplotype were analyzed for this fragment. The 40 resulting sequences were of 569 bp and corresponded to two haplotypes. The nucleotide diversity of the I3-M11 partition ($\pi = 0.00386$) was higher than that of the 5′-end partition ($\pi = 0.00058$), indicating better resolution at the intraspecific level. Sponges with the most divergent external morphologies (smooth vs. digitate surface) had different haplotypes, while those with the most common external morphology (rough surface) presented a mixture of haplotypes. Pairwise tests for genetic differentiation among geographic locations based on F_{ST} values showed significant genetic divergence between most populations, but this genetic differentiation was not due to isolation by distance. While limited larval dispersal may have led to differentiation among some of the populations, the patterns of genetic structure appear to be most strongly related to patterns of ocean currents. Therefore, hydrological features may play a major role in sponge colonization and need to be considered in future plans for management and conservation of these important components of coral reef ecosystems.

Keywords Sponge · *Xestospongia muta* · Population genetics · Genetic structure · mtDNA · Ocean currents

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Introduction

Sponges are a prominent component of Caribbean coral reefs (Diaz and Rützler 2001). In terms of species richness, abundance, and biomass, sponges rival both hard and soft corals (Targett and Schmahl 1984; Diaz and Rützler 2001). In addition, sponges provide refuge for many small invertebrates, are critical to benthic-pelagic coupling, have symbiotic associations with microorganisms (Diaz and Rützler 2001), and produce secondary metabolites with interesting ecological and pharmaceutical properties (reviewed in Paul and Ritson-Williams 2008). Sponge larvae can disperse over short (e.g., Uriz 1982; Uriz et al. 1998; Mariani et al. 2000) or long distances (e.g., Maldonado and Uriz 1999; Vacelet 1999; Lazoski et al. 2001), depending on the species. Gene flow is often correlated with larval dispersal capabilities and population structure, yet the existing molecular studies of sponges have mainly focused on resolving the taxonomically problematic groups (reviewed in Knowlton 2000).

Although the ecological importance of sponges is well established, there have been few studies of the genetic

variation among sponge populations and spatial patterns of this variation due to a lack of variable single-copy markers with enough resolution to differentiate populations (Wörheide et al. 2004, 2005). Microsatellite loci are currently available for only three sponge species (Duran et al. 2002; Knowlton et al. 2003; Blanquer et al. 2005), and other nuclear markers present low nucleotide variability even at the species level (Blanquer and Uriz 2007) or present intragenomic variation that makes them unsuitable for analyses at the population level (nrDNA ITS region; Duran et al. 2004a; Nichols and Barnes 2005). Recently, the second intron of the nuclear ATP synthetase beta subunit gene (ATPSbeta-iII) has been used to study the population structure in the two sponges: *Pericharax heteroraphis* and *Leucetta chagosensis* (Bentlage and Wörheide 2007; Wörheide et al. 2008). Although these results were promising and showed an increased intraspecific diversity when compared to other genetic markers, some potential limitations, including false heterozygotes, the large effective population size of bi-allelic nuclear DNA, and potential intragenic recombinations, need to be considered (Moore 1995; Posada and Crandall 2002; Nichols and Barnes 2005; Bentlage and Wörheide 2007).

Mitochondrial genes remain the preferred choice for population genetic and phylogeographic studies of marine organisms because they are maternally inherited without recombination, have shorter coalescence times, and are expected to undergo lineage sorting three times faster than nuclear markers (Avise et al. 1987; Palumbi et al. 2001). However, few studies have attempted to determine the genetic structure of sponge populations using mitochondrial markers (Duran et al. 2004b; Wörheide 2006; Duran and Rützler 2006), and these studies targeted a fragment of the cytochrome *c* oxidase subunit I (COI) gene amplified with the universal primers of Folmer et al. (1994; hereafter called as 5′-end partition). The results of these previous studies revealed low levels of genetic variation between populations, including those separated by distances of more than 7,000 km (Duran et al. 2004b; Wörheide 2006). In a recent study, Erpenbeck et al. (2006) found that the COI I3-M11 partition in sponges presented more variability than the 5′-end partition at the species level and, therefore, seemed to be more suitable for taxonomic studies and DNA bar-coding. To date, however, no study has attempted to determine the usefulness of the I3-M11 partition as a genetic marker to assess the population genetic structure of sponges.

The giant barrel sponge, *Xestospongia muta* (Demospongiae: Haplosclerida) is a large and common member of Caribbean coral reef communities. Populations of this species occupy greater than 9% of the available reef substrate in some regions (Zea 1993). On the reefs off Key Largo, Florida, mean densities of *X. muta* are ~0.2 sponges m^{-2} (McMurray et al. 2008), and the biomass of this species exceeds that of any other benthic invertebrate. Individuals are often very large, with heights and diameters in excess of 1 m, and some of these large individuals are estimated to be >1,000 years old (McMurray et al. 2008). Individuals of *X. muta* are highly variable in size, shape, and external surface morphology, with the last of these varying from smooth to highly digitate or lamellate (Kerr and Kelly-Borges 1994). Past studies of the sterol composition of the tissues of *X. muta* have established that distinct chemotypes exist (Fromont et al. 1994), but they did not correlate with geographic locality, depth, microhabitat, or morphotype (Kerr and Kelly-Borges 1994).

Tissues of *X. muta* contain cyanobacterial symbionts belonging to the *Synechococcus* group (Gómez et al. 2002; Steindler et al. 2005). Like reef-building corals, *X. muta* is subject to occasional bleaching (loss of the reddish-brown coloration; Vicente 1990). Two types of bleaching have been described: cyclic bleaching, from which sponges recover, and fatal bleaching, which often results in sponge death (Cowart et al. 2006). Reports of sponge bleaching and disease have increased dramatically in recent years and have been observed throughout the Caribbean (reviewed in Webster 2007). In a recent study of stress proteins in *X. muta*, López-Legentil et al. (2008) concluded that, as amply demonstrated for corals, an increase in seawater temperature may result in a decline of *X. muta* populations in the Caribbean. In addition, elevated seawater temperatures may affect the frequency and severity of disease outbreaks by increasing the prevalence and virulence of pathogens, facilitating new invasions or reducing host resistance and resilience (Sutherland et al. 2004). Therefore, sponge populations are threatened by both global warming and disease. In order to assess the sponge population vulnerability, it is important to understand how populations are connected and to determine their genetic diversity. Knowledge of marine population connectivity and larval dispersal is critical to understand past impacts and future prospects for conservation, and to design appropriate management plans for coral reef ecosystem biodiversity (Jones et al. 2007).

In this study, the variation of the I3-M11 partition of the mitochondrial COI gene was analyzed for 116 individuals from seven populations to determine the genetic structure of *X. muta* in Florida, the Bahamas, and Belize. These specimens presented the typical vase-shaped morphology with a highly irregular surface. In addition, to further test the usefulness of the I3-M11 partition at lower taxonomic levels, 10 specimens of each haplotype detected with the I3-M11 partition were sequenced for the 5′-end partition using Folmer et al. (1994) universal primers. Finally, eight specimens presenting the most divergent morphologies (four with pronounced digitate extensions and four with smooth surfaces) were sequenced to assess whether morphological differences could be related to genotype. The main goal of

this study was to determine the appropriateness of the I3-M11 partition to establish genetic diversity baselines and to assess the degree of connectivity and gene flow between populations using *X. muta* as a sponge model.

Materials and methods

Samples

Specimens of *X. muta* (Demospongidae; Haplosclerida) were collected from seven locations within the Caribbean biogeographic region: Key Largo (Florida), Belize, and Sweeting's Cay, Plana Cay, San Salvador, Little San Salvador, and Stirrup Cay (Bahamas; Fig. 1, Table 1). Specimens presenting less common morphologies were photographed and analyzed separately to check for genetic particularities: four specimens with smooth external surfaces and four specimens with pronounced digitate projections on their external surfaces (Figs. 2, 3, respectively). The sampling was undertaken in 2006 and 2007 by SCUBA diving.

DNA extraction and sequencing

Samples were kept in absolute ethanol or frozen at −25°C until used. Mitochondrial DNA was extracted using the Puregene kit (Gentra Systems). The primers C1-J2165 and C1-Npor2760, described in Erpenbeck et al. (2002), were used to amplify the COI mitochondrial gene partition I3-M11. Amplification was performed in a 25 µl total-reaction volume with: 1.25 µl of each primer (10 µM), 0.5 µl dNTP's (10 mM), 2.5 µl 10× buffer, 2 µl $MgCl_2$, 0.5 µl Taq polymerase 5U, and 1 µl DNA. A single soak at 95°C for 5 min was followed by 35 amplification cycles (denaturation at 95°C for 30 s; annealing at 42°C for 30 s; and extension at 68°C for 1 min 30 s), and a final extension at 72°C for 10 min, in a Peltier PTC-200 gradient PCR.

The PCR products were purified using the Qiagen PCR purification kit. The sequencing reaction was carried out with the BigDye TM terminator v. 3.1 using the same primers as in the amplification step. Sequences were obtained on an ABI Prism 3100 automated sequencer. The best match from BLAST searches of GenBank was with a sequence from *Xestospongia proxima* (99% identity; AM076980; Rot et al. 2006).

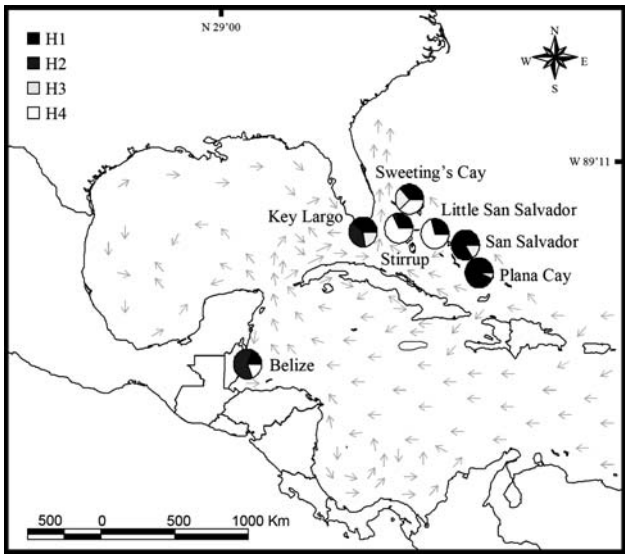

Fig. 1 Map showing the seven sampling sites of *Xestospongia muta* in Northern Caribbean: Key Largo, Belize, Sweeting's Cay, Plana Cay, San Salvador, Little San Salvador, and Stirrup Cay. The haplotype frequencies for each population are depicted in pie charts. Main surface currents affecting the study area are depicted as compiled by Roberts (1997). Scale in kilometers

The universal primers LCO1490 and HCO2198, described in Folmer et al. (1994), were used to sequence 10 specimens for each haplotype obtained with the primers described in Erpenbeck et al. (2002). Specimens for each haplotype were selected at random. Amplification, PCR product purification, and sequencing were performed as indicated above.

Population genetics

Sequences were aligned using Bioedit version 7.0.5.2 (Hall 1999) and alignments were confirmed by eye. Further anal-

Table 1 Diversity measures for the populations of *Xestospongia muta* studied

Population	GPS coordinates	Pc	*n*	*p* (SD)	*h* (SD)	H1	H2	H3	H4
Key Largo	24°57′13″N; 80°27′13″W	1	18	0.0023(0.0017)	0.6863(0.0499)	0.389	0.389	0.222	0
Belize	17°21′10″N; 87°45′36″W	2	16	0.0033(0.0023)	0.575(0.112)	0.188	0.625	0	0.188
Sweeting's Cay	26°36′05″N; 77°52′58″W	3	19	0.0036(0.0024)	0.6959(0.0417)	0.368	0	0.263	0.368
Plana Cay	22°36′27″N; 73°37′34″W	4	15	0.0005(0.0006)	0.1333(0.1123)	0.933	0	0.067	0
San Salvador	24°03′23″N; 74°32′31″W	5	12	0.0011(0.0011)	0.303(0.1475)	0.833	0	0.167	0
Little San Salvador	24°34′27″N; 75°57′28″W	6	14	0.0032(0.0022)	0.4396(0.112)	0.286	0	0	0.714
Stirrup Cay	25°49′36″N; 77°53′58″W	7	22	0.0035(0.0023)	0.4805(0.0935)	0.273	0.045	0	0.682

Population GPS coordinated, population code (Pc), population sample size (*n*), nucleotide diversity (*p*), haplotype diversity (*h*) and haplotype frequencies (H1, H2, H3 and H4). Standard deviations (SD) are given in parentheses

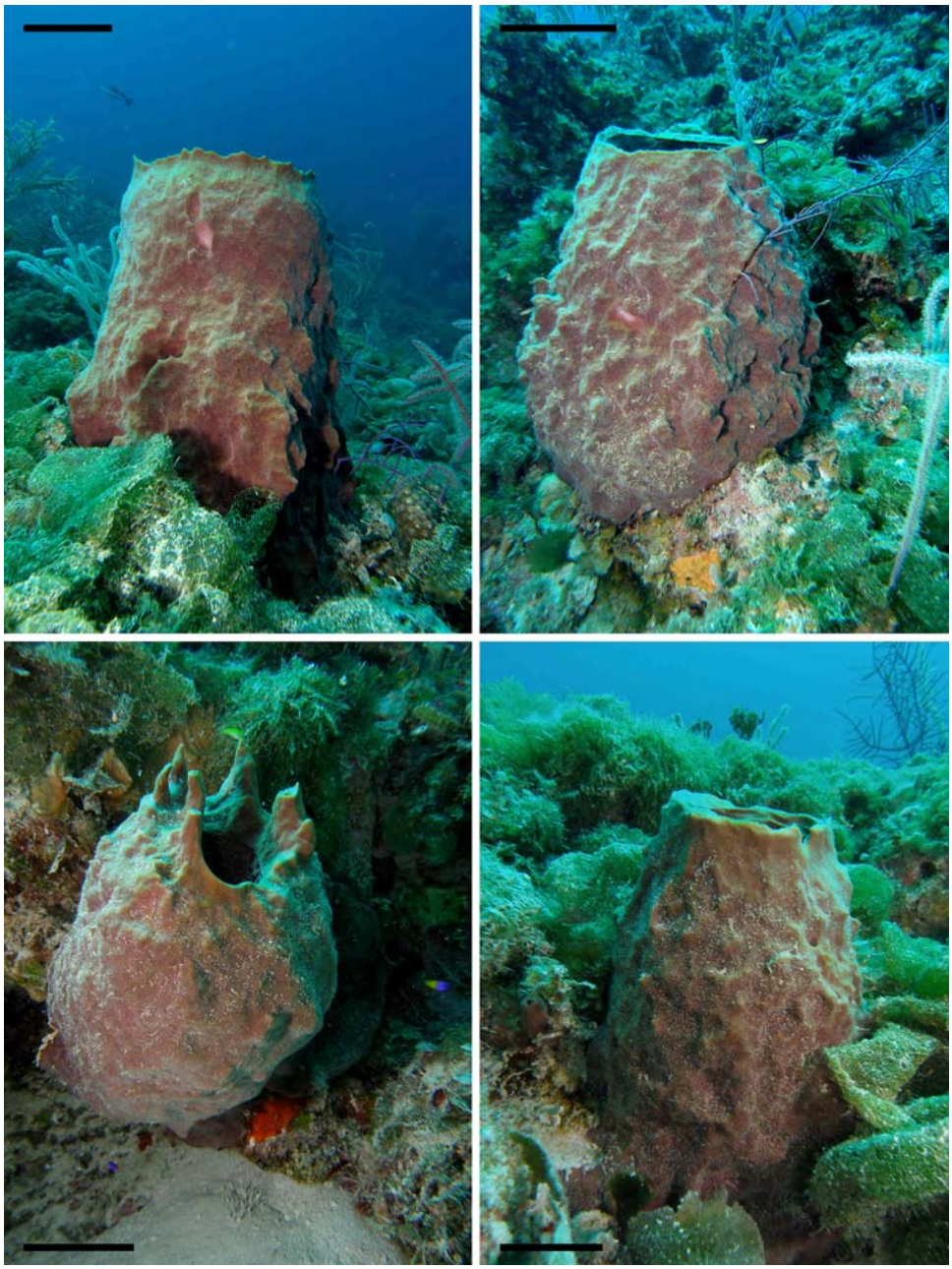

Fig. 2 Smooth surface morphotypes of *Xestospongia muta* from Sweeting's Cay presenting haplotype 4 (H4). Scale bar = 4 cm

yses were performed using Arlequin v. 2000 (Schneider et al. 2000). Nucleotide diversity and haplotype diversity (Nei 1987) were calculated for each population. The exact test of population differentiation (Raymond and Rousset 1995) was used to test the null hypothesis of a random distribution of the different haplotypes among geographic locations. The significance was estimated from 10,000 random permutations of the original data matrix. Pairwise F_{ST} values and their significance were calculated using permutation tests (10,000 replicates) among pairs of populations. A Mantel test with 10,000 permutations was performed to test the isolation by distance between populations (Rousset 1997). Analysis of molecular variance (AMOVA; Excoffier et al. 1992) was performed to examine the hierarchical population structure.

A multidimensional scaling analysis (MDS) was also performed on a matrix of pairwise genetic distances based on the F_{ST} values. The MDS was run in two dimensions with the Kruskal linear algorithm using the statistical software Systat v. 9. In order to assess the significance of major MDS groups, the ANOSIM test was performed using PRIMER v. 5.0.

In order to assess the relationships between haplotypes, a haplotype network was estimated using the TCS v 1.21

Fig. 3 Morphotypes with digit-like surface extensions of *Xestospongia muta* from Sweeting's Cay presenting haplotype 3 (H3). Scale bar = 4 cm

Table 2 Nucleotide differences between the four haplotypes obtained for the I3-M11partition of COI and the two haplotypes obtained for the 5′-end partition

I3-M11 partition	Variable Site 1	Variable Site 2	Variable Site 3	Variable Site 4	Variable Site 5
COI Position	766	777	783	888	1102
Haplotype 1 (H1)	A	T	C	A	G
Haplotype 2 (H2)	A	T	T	A	G
Haplotype 3 (H3)	A	A	C	G	G
Haplotype 4 (H4)	G	A	C	G	A
5′End partition	Variable Site 1				
COI Position	371				
Haplotype 1	C				
Haplotype 2	T				

The COI absolute position of each variable site was determined based on the complete mitochondrial sequence of *Xestospongia muta* (Kayal and Lavrov 2008)

program (Clement et al. 2000). This method estimates an unrooted tree and provides a 95% plausible set for parsimonious relationships between the haplotypes.

Results

In total, 116 partial sequences of the I3-M11 partition of the COI mitochondrial gene were obtained from seven populations of the giant barrel sponge *X. muta* (Table 1). After alignment and trimming, a final sequence length of 544 bp was used in subsequent analyses. Diversity measures for the studied populations of *X. muta* are summarized in Table 1. Four haplotypes were found (Fig. 1; GenBank accession no.: EU716652 to EU716655), with a total of five (0.92%; Table 2) variable sites and overall nucleotide diversity $\pi = 0.00386$.

Haplotype 4 (H4) was found in the four specimens having a smooth exterior surface (Fig. 2), while Haplotype 3 (H3) was found in the four specimens having long digitate processes on their surface (Fig. 3). Although these two morphotypes had different haplotypes, the same two haplotypes were interspersed among specimens with the more common rough exterior surface. Haplotype 1 was present in

Table 3 Pairwise F_{ST} values between populations of *Xestospongia muta*

Population	Key Largo	Belize	Sweetings Cay	Plana Cay	San Salvador	Little San Salvador	Stirrup Cay
	1	2	3	4	5	6	7
1	0						
2	0.07712	0					
3	0.13420*	0.26049*	0				
4	0.32401*	0.56499*	0.32837*	0			
5	0.20569*	0.46626*	0.20776*	−0.02767	0		
6	0.35862*	0.37295*	0.09443	0.61454*	0.50833*	0	
7	0.33922*	0.33820*	0.09504	0.55995*	0.47364*	−0.05809	0

Significant values at $P < 0.05$ are indicated with an asterisk

Table 4 Hierarchical analysis of molecular variance (AMOVA) based on the sequences obtained for the 5′-end partition of the cytochrome c oxidase subunit I (COI) of *Xestospongia muta*

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among populations	6	12.58	0.11228 Va	31.26
Within populations	109	26.91	0.24690 Vb	68.74
Total	115	39.49	0.35918	
Fixation index F_{ST}	0.31261*			

Significant values at $P < 0.05$ are indicated with an asterisk. Va, Vb and Vc are the associate covariance components. F_{ST} is the fixation index

all the studied populations and was shared by 51 individuals, whereas haplotype H3 was found in Key Largo, Sweeting's Cay, Plana Cay, and San Salvador, and was only found in 12 specimens.

The results of the exact test for population differentiation, based on the haplotype frequencies between the different locations, revealed an overall significant heterogeneity in the distribution of haplotypes ($P < 0.05$). Pairwise tests for genetic differentiation among geographic locations based on F_{ST} values showed significant genetic divergence between most population-pairs. Some exceptions were: Key Largo compared with Belize; Sweeting's Cay compared with Little San Salvador and Stirrup Cay; Plana Cay compared with San Salvador; and Little San Salvador compared with Stirrup Cay (Table 3). There was no evidence of isolation by distance between the populations studied (Mantel test, $P = 0.4159$). In fact, although Belize was the most distant population (over 1,000 km from the others), and was genetically different from all the Bahamas populations, populations much closer were also genetically different (e.g., Key Largo and Sweeting's Cay, separated by less than 100 km). Likewise, San Salvador and Little San Salvador (154.5 km apart) were also genetically differentiated.

The hierarchical AMOVA analysis revealed that 31.26% of the genetic variance was found among populations. A greater percentage of variance (68.7%) was explained by comparisons within populations (Table 4). Both components were highly significant in a permutation test ($P < 0.0001$). The overall F_{ST} value (0.3126) was greater than those obtained from random permutations of haplotypes between populations ($P < 0.0001$), which indicates that there was a strong genetic structure in the study area.

Populations of *X. muta* were well separated in the two-dimensional space of the MDS ordination based on the matrix of F_{ST} distances (stress of the configuration <0.001). Sweeting's Cay, Stirrup Cay, and Little San Salvador grouped together; Plana Cay and San Salvador formed another group; and Belize and Key Largo grouped closer together than with the others (Fig. 4). The ANOSIM test

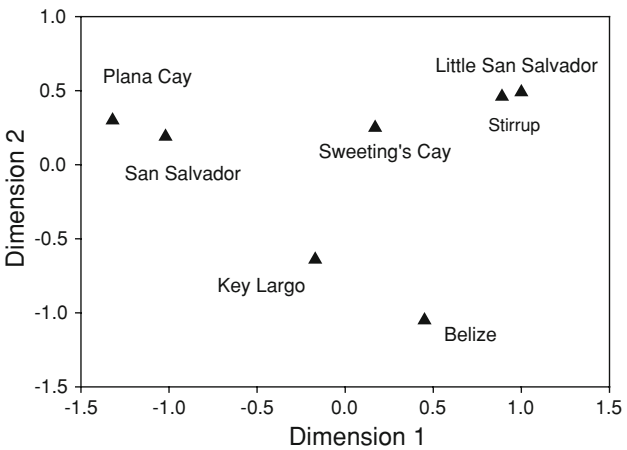

Fig. 4 MDS analysis results based on the pairwise F_{ST} distances between *Xestospongia muta* populations in Northern Caribbean

revealed a significant structure in our data ($P = 0.01$). However, the significance of pairwise comparisons could not be assessed due to the low number of possible permutations.

The parsimony haplotype network is presented in Fig. 5. The single most common haplotype (square box) corresponded to Haplotype 1. Haplotype 2 was directly connected to haplotype 1 through one mutational step, while haplotypes 3 and 4 where indirectly connected to haplotype 1 through mutation steps 2 and 4, respectively.

The 5′-end partition of the COI gene of 40 specimens resulted in nucleotide sequences of 569 bp in length. From these samples, which include individuals from all of the populations studied, two haplotypes were identified. The first haplotype was found in eight specimens that had haplotype H3 for the I3-M11 partition of COI (GenBank accession no. EU716650). All the remaining sequences (32) presented the second haplotype (GenBank accession no. EU716651) and these corresponded to individuals that had haplotypes H1, H2, or H4 for the I3-M11 partition. There was only one nucleotide change between the two 5′-end haplotypes (0.18% variable sites; Table 2) and the nucleotide diversity was $\pi = 0.00058$.

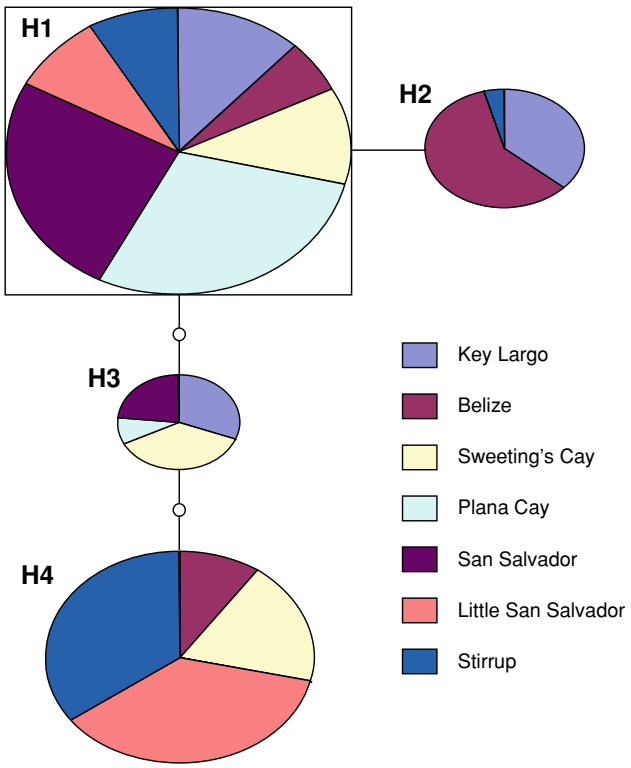

Fig. 5 *Xestospongia muta* haplotype network based on the I3-M11 partition of COI. Sizes of ovals are proportional to the frequency of haplotypes and the central rectangle represents the single most common haplotype. Proportions of colors within each pie chart are representative of the relative number of that haplotype from each population. Empty circles represent inferred haplotypes that were not sampled. Each line segment in the network represents a single nucleotide substitution

Discussion

Analysis of mtDNA sequences of the sponge *X. muta* revealed that the COI partition I3-M11 yielded a higher nucleotide and haplotype diversity than did the 5′-end COI partition in sponges ($\pi = 0.0006$, Duran et al. 2004b; $\pi = 0.00049$, Wörheide 2006; $\pi = 0.00058$, this study). In addition, the nucleotide diversity reported here for the I3-M11 partition ($\pi = 0.00386$) is within the range found for the 5′-end partition in other invertebrates with lecithotrophic larvae (e.g., ascidians $\pi = 0.0018$–0.0032, Tarjuelo et al. 2001). Significant F_{ST} values were found for the majority of population comparisons, indicating that there is a clear genetic structure among the studied populations. As found in the few studies addressing the genetic structure of marine invertebrates in the Caribbean (Mitton et al. 1989; Shulman and Bermingham 1995), there was no evidence of isolation by distance acting on populations of *X. muta*.

Roberts (1997) predicted a strong influence of surface currents in larval dispersal and genetic structure of populations in the Caribbean. Indeed, surface currents are vectors of gene flow determining the genetic structure for marine species with dispersive larvae (e.g., Hateley and Sleeter 1993; Silberman et al. 1994; Shulman and Bermingham 1995; Galindo et al. 2006). The dispersal distance and time for sponge larvae are mostly unknown, and likely vary depending on the species (Uriz 1982; Uriz et al. 1998; Maldonado and Uriz 1999; Vacelet 1999; Mariani et al. 2000). Although no information is available for the larval life-span of *X. muta*, the pattern of currents in the Caribbean seems to play an important role in determining the genetic disparities found among populations. For instance, larval transport in the strong Caribbean Current may explain the lack of genetic differentiation between Belize and Key Largo, 1,133 km apart. Taylor and Hellberg (2003), and Colin (2003) reported a central-northern Bahamas barrier in fish larval dispersion. Central Bahamas populations in that study included samples from San Salvador, while some samples from Northern Bahamas were collected in Sweeting's Cay. Gutiérrez-Rodríguez and Lasker (2004) reported significant genetic distance between San Salvador and Little San Salvador populations of the gorgonian *Pseudopterogorgia elisabethae.* Additionally, in an oceanographic-genetic model for the Caribbean, Galindo et al. (2006) predicted genetically dissimilar populations of corals between the Northern and the Southern islands of the Bahamas based on the current patterns between the islands. Likewise, in the present study, a genetic barrier between Northern Bahamas (Stirrup Cay, Sweeting's Cay, and Little San Salvador) and Central Bahamas (San Salvador and Plana Cay) was detected between populations of *X. muta*. While limited dispersal may have led to differentiation among some close populations, the patterns of genetic

similarity found here appear to be related to main ocean currents in the Caribbean and within the Bahamas (Fig. 1).

In this study, two different morphotypes of *X. muta* (smooth, Fig. 2; and with pronounced digitate projections, Fig. 3) were found to have different genotypes (H4 and H3, respectively). However, haplotype H3 and H4 were also found in specimens presenting the typical vase-shaped morphology with an irregular, rough surface, suggesting that hybridization occurs between these forms. Kerr and Kelly-Borges (1994) previously found that the exterior morphology of *X. muta* did not correlate to a potential chemotaxonomic indicator (sterol chemotypes), nor did morphology or chemotype correlate with geographic locality, depth, or microhabitat. It remains to be determined whether there is a relationship between the distinct sterol chemotypes found in *X. muta* (Fromont et al. 1994) and the haplotypes described in the present study.

Although the haplotype diversity found here was low when compared with other animal groups, the I3-M11 partition of COI is presently the best available mitochondrial marker to address population structure questions. The I3-M11 partition has better resolution and higher nucleotide diversity than the 5′-end partition at the intraspecies level; a conclusion that was also reached by Erpenbeck et al. (2006) at the interspecies level. Our data reveal strong genetic structure among proximate populations, indicating restricted gene flow among them. In addition, results are in accordance with the few previous studies of genetic structure among Caribbean marine invertebrates, suggesting that oceanic currents play a key role in dispersal and connectivity; hence their importance in the design and management of conservation areas associated with coral reef ecosystems.

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