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Morphological and genetic evaluation of the hydrocoral *Millepora* species complex in the Caribbean

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Abstract

Background: The hydrocoral *Millepora* is an important framework builder that dominates shallow turbulent environments in the Indo-Pacific and the Atlantic-Caribbean. The Caribbean representatives of the genus are classified in four species - *Millepora alcicornis*, *Millepora complanata*, *Millepora striata*, and *Millepora squarrosa* - but their taxonomic boundaries are not clearly defined. We used mitochondrial gene sequences to delineate the four *Millepora* species and evaluated whether morphological traits and mitochondrial sequence divergence were correlated for two most common species *M. alcicornis* and *M. complanata*.

Results: Samples were collected from Puerto Rico, Guadeloupe, Curaçao, Grand Cayman, and Panama during 2006 to 2007. Diameter of dactylopores distinguished the branching and encrusting morphotypes of *M. alcicornis* and *M. complanata*, and gastropore diameter discriminated between *M. alcicornis* and *M. complanata*. High levels of haplotypic diversity ($H_d = 0.94$) were observed, with the most common haplotypes shared by *M. alcicornis* and *M. complanata*. Sequence divergence ranged from 0% to 3% among *M. alcicornis*, *M. complanata*, and *M. striata* to 25% between these three species and *M. squarrosa*. Bayesian analysis of cytochrome oxidase subunit I (COI) gene indicated the presence of three Caribbean taxa: *M. squarrosa*, *M. striata*, and the 'species complex' encompassing the morphologies displayed by *M. complanata* and *M. alcicornis*.

Conclusions: The branched *M. alcicornis* and encrusted *M. alcicornis* and *M. complanata* can be differentiated morphologically but not genetically. Phylogenetic analysis suggests that the Caribbean milleporids include three species - *M. squarrosa*, *M. striata*, and the species complex of *M. alcicornis-M. complanata*. *Millepora striata* is closely related to the *M. alcicornis-M. complanata* species complex.

Keywords: Hydrozoan; Polymorphism; Phenotypic plasticity; Cytochrome oxidase I; Puerto Rico

Background

The hydrocoral genus *Millepora* consists of 19 species distributed in warm waters around the globe. Twelve of these are found in the Indo-Pacific and seven in the Atlantic-Caribbean, with no common species between the two oceans (Boshma 1948; Cairns 1999; Amaral et al. 2008). Some *Millepora* species are important framework builders in some locations as they dominate shallow turbulent environments, forming dense reef rims contributing to the stabilization and complexity of the carbonate structure (Lewis 1989; Edmunds 1999). Additionally, *Millepora* are voracious plankton feeders, consuming up to 8 prey cm^{-2} day^{-1} (Lewis 1992).

Milleporids are superior space competitors over gorgonians (Wahle 1980) and are immune to predation by the starfish *Acanthaster planci* (Lewis 1989). However, *Millepora* is one the first cnidarians to lose its zooxanthellate symbionts during bleaching events (Glynn 1993; Paulay and Benayahu 1999; Marshall and Baird 2000). Despite their abundance, geographical distribution, and geological importance, the milleporids have seldom received attention in coral reef studies (Lewis 1989). Similar to some groups of scleractinian corals, lack of information is partly due to the high degree of morphological variation within the genus, which renders identification of species difficult.

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The taxonomic status of the various *Millepora* species has been controversial for more than three and a half centuries (Boshma 1948; Manchenko et al. 1993). Efforts to identify species were mainly based on morphological characters of the corallum (the calcium carbonate skeleton): presence or absence of ampullae (receptacles bearing the sexual medusa), texture of the colony, and number of gastropores and dactylopores (Boshma 1948; de Weerdt 1984; Razak and Hoeksema 2003). However, due to their high morphological variability, disagreement exists on the species classification based on those traits (de Weerdt 1984; Lewis 1989; Amaral et al. 2002). Accepted valid species are those described by Boshma (1948) and later revisited by Cairns (1999); however, the taxonomic revision of the genus is still in progress. New forms of milleporids are being described and proposed as new species (Amaral et al. 2002, 2008), while a more recent revision examined the range of morphological variation and the validity of the taxonomical characters (Razak and Hoeksema 2003).

Seven species of *Millepora* are described from the western tropical Atlantic: *Millepora alcicornis* Linnaeus 1758, *Millepora complanata* Lamarck 1816, *Millepora squarrosa* Lamarck 1816, *Millepora striata* Duchassaing and Michelotti 1864, *Millepora nitida* Verrill 1868, *Millepora braziliensis* Verrill 1868, and *Millepora laboreli* Amaral et al. 2008; the last three are restricted to Brazil. The species

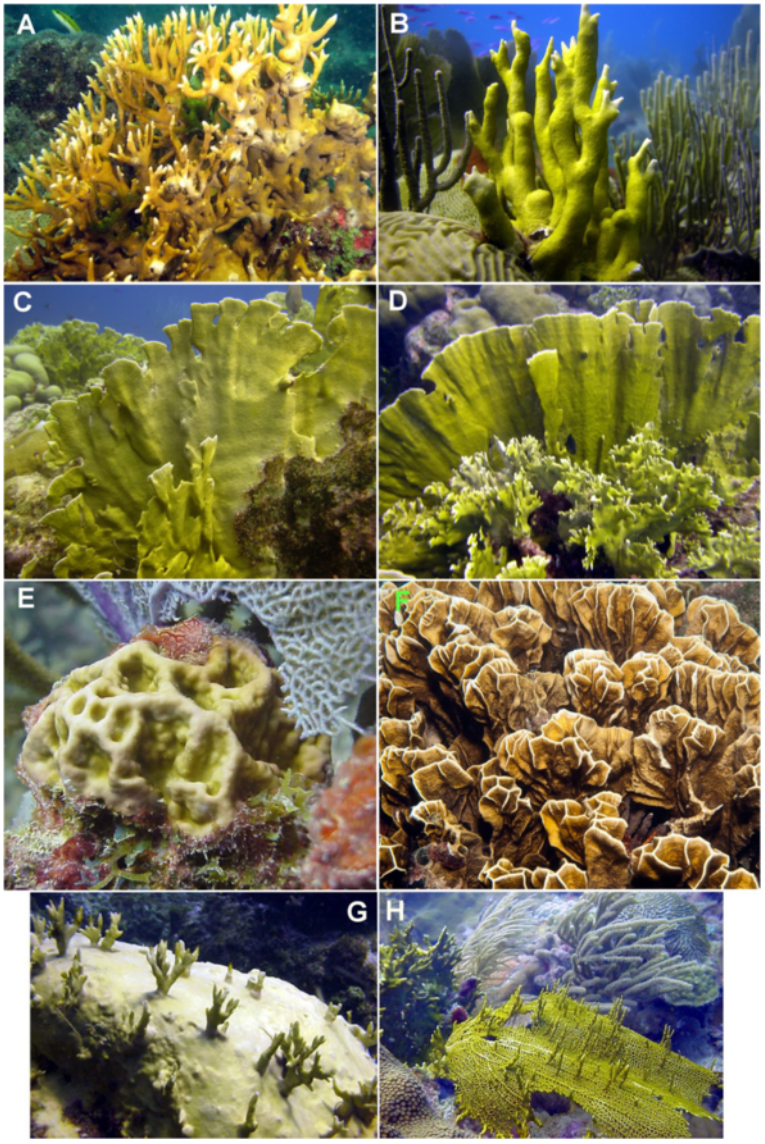

Figure 1 The different described species of *Millepora* in the Caribbean. Typical *M. alcicornis* colony from Puerto Rico **(A)**, and a different thicker ecomorph (or a potentially different taxon) common in Bermuda **(B)**. A typical colony of *M. complanata* **(C)**, and *M. alcicornis* (front) and *M. complanata* (back) coexisting in the same habitat in Curaçao **(D)**. Medium size *M. squarrosa* in Puerto Rico **(E)**, and the rare *M. striata* from Honduras **(F)**. Several different ecomorphs of *Millepora* (some crustose aggressive colonizers of substrate) **(G)**, and some killing and colonizing octocorals **(H)**. (Photos by E. Weil)

in this study included *M. alcicornis*, *M. complanata*, *M. squarrosa*, and *M. striata* (Additional file 1, Figure 1).

M. alcicornis is abundant in the Caribbean, Bermuda, Brazil, and West Africa, primarily at less exposed areas of reefs and lagoons (de Weerdt 1984; Lewis 2006). Colonies are branching; branches may be very delicate or coarse and largely united into plate-like constructions with flattened growing edges. The corallum grows mostly upright, but it may also encrust other sessile organisms and hard substrates. The surface of the colony is smooth and even, and the gastropore size ranges from 0.15 to 0.30 mm, while the dactylopores are small (0.06 to 0.17 mm) (de Weerdt 1984).

M. complanata is also common throughout the Caribbean (de Weerdt 1984; Lewis 2006), inhabiting the surf zone and reef flats. The colonies are formed by simple plates growing from a common base or may create complex honeycomb-like structures of interconnected plates. The plates grow perpendicular to the direction of the current, but in places with strong wave action, colonies may remain as a large encrusting base. The surface of the colonies varies from smooth to rough. The gastropores are large (0.22 to 0.36 mm), and dactylopores are variable in size (0.12 to 0.24 mm).

M. squarrosa is restricted in the northeastern, eastern, and southeastern Caribbean and is also reported for Brazil (de Weerdt 1984, 1990). The colonies consist of irregular, heavy-connected, thick masses with smooth rounded edges. In older colonies, the growth form can become more plate-like and the growing edges sharper, but the plates remain connected along their whole length, resulting in a box-work structure. The surface is smooth but irregular due to the presence of crests and tubercles. Gastropore size varies from 0.20 to 0.30 mm, and the dactylopores vary from 0.07 to 0.15 mm.

M. striata is reported from Panama, Colombia, Venezuela and Guadeloupe (de Weerdt 1984), and Belize (Fenner 1999). Colonies are formed by loosely connected plates of very sharp edges with a strong tendency to divide along the upper edge. Longitudinal folds make the surface very uneven. Gastropores grow to sizes from 0.15 to 0.25 mm, and dactylopores vary from 0.08 to 0.18 mm.

The few taxonomical studies on the Caribbean *Millepora* highlight the high degree of skeletal polymorphism encountered in the genus. Two previous studies (Martínez-Estalella 1982; de Weerdt 1984) concluded that species distinctions were uncertain due to the high morphological variability among the colonies within each taxon and the overlap of skeletal characteristics between species. Additionally, reciprocal transplantation experiments (de Weerdt 1981) suggested that environmental factors might influence the colony shape. Even though controversy persists around the genus *Millepora*, little has been done to unravel its taxonomic ambiguity by using multicharacter approaches including molecular techniques, such as those used with scleractinian corals (Weil and Knowlton 1994). The few available studies focused on species from Vietnam, the Red Sea, Brazil, and Bahamas (Manchenko et al. 1993; Meroz-Fine et al. 2003; Amaral et al. 2008; Squiers et al. 2011).

The purpose of this study was to combine a morphologically based *Millepora* classification scheme with new molecular information based on the mitochondrial gene cytochrome oxidase subunit I (COI) to test whether different morphotypes of *M. alcicornis* and *M. complanata* represent genetically distinct taxa. The four *Millepora* species were evaluated genetically in an attempt to clarify the status of the *Millepora* species reported from Caribbean waters.

Methods

Sampling methods

Samples were collected from the fringing reefs of Turrumote (17.934°N, 67.018°W), Media Luna (17.938°N, 67.041°W), Enrique (17.954°N, 67.051°W), Las Pelotas (17.956°N, 67.073°W), and Margarita (17.922°N, 67.097°W) in La Parguera, southwest coast of Puerto Rico (PR) (Additional file 2) at depths between 1 and 15 m. *M. squarrosa* was collected at Media Luna and Turrumote reefs at depths of 15 m. All samples were collected during 2006 to 2007. Colonies were classified as *M. alcicornis*, *M. complanata*, *M. squarrosa*, or *M. striata* (Additional file 1, Figure 1), using the most recent taxonomic descriptions for the Caribbean species (de Weerdt 1984). In addition, sequences of *M. alcicornis* and *M. complanata* from seven localities across the Caribbean were added for genetic analysis: Bocas del Toro, Panama (9.231°N, 82.137°W); Grand Cayman; Mona, Puerto Rico (PR) (18.105°N, 67.940°W); La Parguera, PR (17.970°N, 67.046°W); Vieques, PR (18.119°N, 65.577°W); Guadeloupe; and Curaçao (12.084°N, 68.896°W). COI sequences from *M. striata* collected from Bocas del Toro, Panama were also included in the analysis.

M. alcicornis was divided into two morphotypes (Additional file 3): free growth (from now on referred as branching) and the encrusting form that covers substrata and octocorals (crustose or laminar). de Weerdt (1984) and Lewis (1989) identified *Millepora* growing on gorgonians as *M. alcicornis*; however, colonies of both *M. alcicornis* and *M. complanata* have been observed overgrowing gorgonians (Wahle 1980; Squiers et al. 2011; all authors, personal observation). There is a wide morphological variability of *Millepora* overgrowing octocoral colonies, but we only included colonies that were tentatively identified as *M. alcicornis* by the gross morphology of the colony (forming delicate branches, instead of plates) to reduce the range of morphotypes considered within the encrusting forms. All these specimens were treated as a distinct morphotype from other *M. alcicornis*.

M. squarrosa was relatively common in Puerto Rico (de Weerdt 1984, 1990), before the 2005 bleaching event (EW,

unpublished data). Surveys after the bleaching event failed to find a single colony in several reefs off La Parguera (EW, unpublished data). Only three colonies were found in three reefs (Turrumote, Media Luna, and Margarita) during the duration of the study (2006 to 2007). Tissue was collected only for genetic analysis, due to the small size of the colonies. Only two of the colonies were used (Turrumote and Media Luna) because the amplification from the Margarita sample was unsuccessful. *M. striata* has not been reported from Puerto Rico; four specimens were obtained from Bocas del Toro, Panama and used in the genetic analysis.

Ten colonies each of the most representative morphotypes of *M. alcicornis* branched (Mab), *M. alcicornis* encrusted (Mae), and *M. complanata* (Mc) were sampled ($N = 30$) from the front reef within a depth range of 0.5 to 9 m, following a linear transect. Selected colonies were spaced at least 5 m from each other to minimize the collection of clones. Two fragments of each colony were collected and preserved in 100% ethanol for micromorphological and genetic analyses.

Morphological variability

The 30 colonies (Mab = 10, Mae = 10, and Mc = 10) used for the morphometric analysis were collected in the reef crest of Enrique cay to ensure that colonies faced similar conditions during life (de Weerdt 1984). A 3-cm^2 fragment of each colony was used for DNA extraction, and the remainder was cleaned with 5% sodium hypochlorite solution, dried, and analyzed under an Olympus SZH10 stereo microscope (Olympus, Tokyo, Japan) following the procedure by Amaral et al. (2002). Five skeletal characters that have been commonly used in previous *Millepora* taxonomic studies were selected for the morphometric analysis: (1) diameter of the dactylopores, (2) diameter of gastropores (de Weerdt 1984; Amaral et al. 2002), (3) distance between the gastropores (Razak and Hoeksema 2003), (4) distance between the dactylopores, and (5) distance from gastropore to the nearest dactylopore (Additional file 4; Table two in Ruiz-Ramos (2009)).

Photographs of the surface of each colony were taken at × 30 magnification with an Olympus C-5050 camera system attached to the Olympus SZH10 stereo microscope and digitally catalogued. The selected traits were measured using SigmaScan Pro software (SPSS Inc.) after calibrating with a stage micrometer calibration slide of 10 μm accuracy.

Thirty measurements were taken from each trait of the three morphotypes (Table two in Ruiz-Ramos (2009)). To reduce intraspecific variation, the measures were taken at the center of the wave-facing side of the colonies. Parametric and non-parametric (when data did not meet the assumptions of equal variances and normality) one-way ANOVAs were used to test the hypothesis that there were no significant morphological differences between the three taxa. A *posteriori* Tukey's test was used to identify the colonies or species that exhibited significantly different traits. A discriminant function analysis (DFA) was used to test the utility of the five morphological characters to distinguish the pre-grouped colonies identified during the collection. The statistical analyses were performed in InfoStat version 2004 (Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina), SigmaStat (SPSS Inc., Chicago, IL, USA), and JMP version 5.0.1 (SAS Institute Inc., Cary, NC, USA). Voucher specimens used in this study have been deposited in the Museum of Marine Invertebrates, Department of Marine Sciences, University of Puerto Rico, Mayagüez.

Genetic analyses

Genetic analyses were performed for the 30 colonies used in the morphological analysis; four samples of *M. striata* and two of *M. squarrosa* were also added (Additional file 1). Tissue was scraped from the skeletal surface of the colony and DNA extracted using the PureGene DNA isolation kit (Gentra Systems, Inc., Minneapolis, MN, USA).

Initial gene amplifications were made with the COI primers designed by Fukami et al. (2004), but they were suboptimal. *Millepora*-specific primers COIF (5′-TAGA ATTAGCTGGGCCAGGA-3′) and COIR (5′-CCTGTC TGTAAGCAGCATGG-3′) were designed from the initial COI sequences using the Primer 3 software. PCR cycling conditions consisted of an initial denaturation of 3 min at 95°C followed by 35 cycles of 15 s at 95°C for denaturation, 30 s at 50°C of annealing, 60 s at 72°C for extension, and a final extension at 72°C for 5 min. Successful PCR reactions were verified by running 5 μl of the amplicon on a 1% TBE agarose gel stained with ethidium bromide. PCR reactions were cleaned of excess dNTPs, primers, and other impurities by enzymatic treatment with the EXOSAP-IT (Affymetrix) method. Sequencing reactions with each of the primers were prepared with the 3.1 BigDye Termination Kit (Applied Biosystems, Foster City, CA, USA) and were loaded in an ABI 3130xl (Applied Biosystems).

DNA sequencing trace files were processed with CodonCode Aligner (CodonCode, Dedham, MA, USA) and exported to MacClade v. 4.05 (Maddison and Maddison 2000) for alignment. The final sequence alignment used for the analyses was 385 bp long. General summary statistics and population analysis were performed with DnaSP (Rozas et al. 2003) (Additional file 5). DNA neutrality tests such as Tajima's D (Tajima 1989) were computed to test for deviation from the neutral model of molecular evolution (Kimura 1968). Numbers of shared mutations, fixed differences, and F_{ST} values were calculated in DnaSP.

Haplotype networks were drawn with TCS 1.21 (Clement et al. 2000). Phylogenetic trees of the samples were constructed using Bayesian inference (BI) and

maximum likelihood. The AIC criterion in ModelTest (Posada and Crandall 1998) was used to identify the best substitution model for the COI sequences. Maximum likelihood (ML) analysis was performed in PAUP 4.10b (Swofford 2002) with the Tamura-Nei substitution model (Tamura and Nei 1993) as suggested by ModelTest, with unequal base frequencies ($A = 0.22$, $C = 0.23$, $G = 0.18$, $T = 0.36$), probability of invariable sites = 0.70, and gamma distribution parameter of $\alpha = 1.768$. The BI analysis was run in MrBayes (Ronquist and Huelsenbeck 2003) using the HKY substitution model and gamma distribution, with four mcmc chain runs for 1,100,000 generations. Topology robustness was evaluated with 100 bootstrap replicates (Felsenstein 1985) and posterior probabilities.

DNA sequences ($n = 274$) of the *Millepora* species and morphotypes sampled across the Caribbean were added to the sequences from the colonies used in the morphological and genetic analyses. An analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was performed to test if the *M. complanata*, *M. striata*, and the two morphotypes of *M. alcicornis* were significantly differentiated within and across populations. A total of seven populations was included in the analysis: Panama (4 Ms-Panama, 10 Mc-Panama, 21 Mab-Panama), Grand Cayman (6 Mc-GC, 7 Mab-GC, 4 Mae-GC), Mona (7 Mc-Mona, 17 Mab-Mona, 20 Mae-Mona), Parguera (24 Mc-Parguera, 56 Mab-Parguera, 33 Mae-Parguera), Vieques (4 Mc-Vieques, 8 Mab-Vieques, 6 Mae-Vieques), Guadeloupe (4 Mc-Gd, 5 Mab-Gd, 2 Mae-Gd), and Curaçao (16 Mc-Curaçao, 14 Mab-Curaçao, 6 Mae-Curaçao), where Ms = *M. striata*, Mc = *M. complanata*, Mab = *M. alcicornis* branched, and Mae = *M. alcicornis* encrusted. AMOVA analysis was performed in Arlequin v3.11 (Excoffier et al. 2005) with 25,000 replications, corrected by the Tamura-Nei + (Γ = 1.8) model of nucleotide substitution. Genetic differences were partitioned among populations, among morphotypes within populations and within the Caribbean. Pairwise genetic comparisons of the morphotypes were corrected with the Tamura-Nei distance. Phylogenetic trees of the Caribbean-wide *Millepora* were constructed by BI analysis using MrBayes (Ronquist and Huelsenbeck 2003) using the HKY substitution model and gamma distribution, with four mcmc chain runs for 1,100,000 generations. Topology robustness was evaluated with 100 bootstrap replicates (Felsenstein 1985) and posterior probabilities. All sequences have been submitted to GenBank [KC570466 - KC570844].

Results and discussion

Results

Variability of skeletal characters

There were significant differences (ANOVA, $p < 0.0001$) in morphological traits among *M. complanata*, *M. alcicornis* branched, and *M. alcicornis* encrusted (Table two in Ruiz-Ramos (2009)). Mean gastropore diameter (in millimeters) was significantly larger (ANOVA, $p < 0.001$) in *M. complanata* (0.25 ± 0.03 mm) compared to *M. alcicornis* (0.20 ± 0.04 mm), but similar between the two morphotypes of *M. alcicornis* (0.20 ± 0.4 mm). *M. complanata* had significantly larger dactylopores (ANOVA, $p < 0.001$) (0.15 ± 0.02 mm) compared to *M. alcicornis* encrusted (0.13 ± 0.02 mm) and *M. alcicornis* branched (0.12 ± 0.02 mm), but there was no significant difference between the two morphotypes of *M. alcicornis*. The diameters of the gastropores and dactylopores of *M. complanata* and *M. alcicornis* were similar to those previously reported by de Weerdt (1984).

Mean distance (in millimeters) between gastropores was significantly (ANOVA, $p < 0.001$) larger in *M. alcicornis* encrusted (1.45 ± 0.43 mm) compared to *M. alcicornis* branched (1.38 ± 0.38 mm), while *M. complanata* had the smallest values (1.10 ± 0.32 mm). The average distance from gastropore to the nearest dactylopore was significantly larger in *M. alcicornis* branched (0.40 ± 0.09 mm) compared to *M. complanata* and *M. alcicornis* encrusted which had similar mean gastropore-dactylopore distances (0.36 ± 0.09 mm) (ANOVA, $p < 0.0001$, Table two in Ruiz-Ramos (2009)).

Variability within morphotypes

Morphometric variation across colonies was significant ($p < 0.001$) for all the traits measured (Figures five, six, seven, eight, and nine in Ruiz-Ramos (2009)). Differences in the diameter of dactylopores and gastropores and in the distances between the pores were observed among colonies of the three morphotypes and also among colonies of the same morphotype. Some colonies with different morphologies (i.e., *M. complanata* and *M. alcicornis* encrusted) showed skeletal traits with similar dimensions.

Mean diameters of gastropores varied from 0.20 ± 0.03 to 0.25 ± 0.03 mm (Figure five in Ruiz-Ramos (2009)). Gastropore diameters overlapped in the two *M. alcicornis* morphotypes and were highly variable in colonies within and between morphotypes. There were significant differences (ANOVA, $p < 0.0001$) among the mean gastropore diameters between colonies within *M. complanata* with values ranging between 0.16 and 0.32 mm (±0.03). The size of the gastropores of colonies MabCO2, MabMg1, MaeLP9, MaeLP10, and MaeMg1 was significantly larger than that of the other *M. alcicornis* colonies and very similar to the gastropore diameters of *M. complanata*.

Dactylopore mean diameter varied from 0.11 ± 0.02 to 0.15 ± 0.02 mm (Figure six in Ruiz-Ramos (2009)). *M. complanata* exhibited the largest dactylopores, while the smaller were observed in the colonies of the branching

M. alcicornis. The encrusted *M. alcicornis* had the most variable dactylopore size, between 0.05 and 0.20 with a mean size of 0.13 ± 0.02. Some colonies, for example, MaeLP2 and MaeLP3, were very similar to *M. alcicornis* branched, while other colonies such as MaeLP9 were more similar to *M. complanata*. However, some colonies of the three morphotypes (McMg9, McE3, MabCO2, MaeLP7, MaeLP10, and MaeT15) showed similar mean diameters (0.13 ± 0.02).

Mean distances between dactylopores (Figure seven in Ruiz-Ramos (2009)) varied from 0.51 ± 0.10 to 0.53 ± 0.16 mm in the majority of the colonies from all the morphotypes, except MabT26 whose average distances were larger than the rest of the colonies.

The mean distances from gastropore to the nearest dactylopore were also variable (Figure eight in Ruiz-Ramos (2009)). There were significant differences among colonies within the morphotypes (ANOVA, $p < 0.001$), but not among all morphotypes ($p \leq 0.05$). For example, colonies of branched *M. alcicornis* were more similar to those of *M. complanata* than they were to those of the encrusted *M. alcicornis* (Figure eight in Ruiz-Ramos (2009)).

Distances between gastropores were less variable among colonies of the same morphology than the aforementioned traits (Figure nine in Ruiz-Ramos (2009)). The mean distance between gastropores was smaller in *M. complanata* (1.10 ± 0.32 mm) than in the *M. alcicornis* morphs. Colonies of branched *M. alcicornis* (1.38 ± 0.39 mm) were also distinct from colonies of encrusted *M. alcicornis* (1.45 ± 0.43 mm); still, overlapping measurements were recorded among some colonies of the two morphotypes.

In the three morphotypes (*M. alcicornis* branched, *M. alcicornis* encrusted, and *M. complanata*), we found colonies in which their micro-morphological traits were more similar to colonies of other morphotypes than to their own.

Variability across morphotypes

Despite some overlap among morphological traits, most characters were diagnostic to differentiate species. The diameters of the gastropores of *M. complanata* were

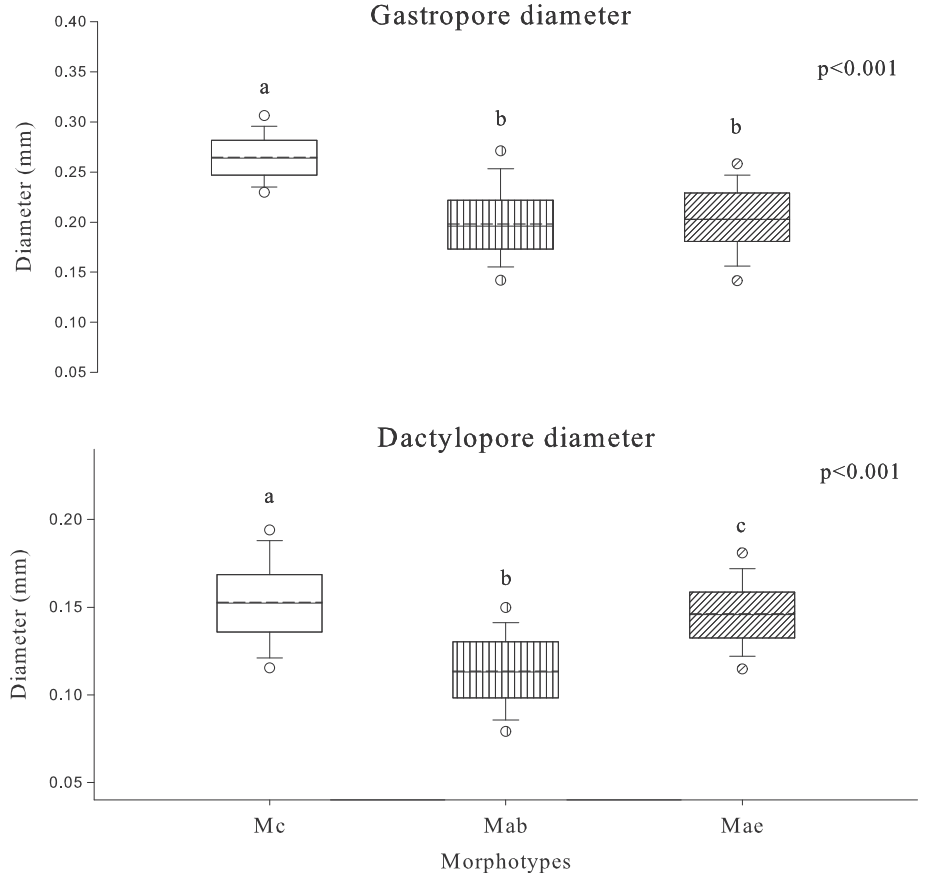

Figure 2 Variability (mean, median, and 5th and 95th percentiles) in the skeletal traits of *Millepora*. The top graph shows variability in gastropore diameter, and the bottom graph shows variability in dactylopore diameters. The solid line is the median, and the dashed line is the mean. The white boxes represent *M. complanata* (Mc), boxes with vertical lines are *M. alcicornis* branched (Mab), and boxes with diagonal lines is *M. alcicornis* encrusted (Mae). Letters above the boxes are values of Tukey's test ($p < 0.05$ for gastropore diameter, $p < 0.001$ for dactylopore diameter); significant differences among morphotypes are denoted by different letters.

significantly larger (Tukey's test, $p < 0.0001$; Figure 2) than in the two morphotypes of *M. alcicornis*. The diameters of the dactylopores were different enough ($p < 0.001$) to distinguish not only between *M. complanata* and *M. alcicornis* but also between the two morphs of *M. alcicornis*. Dactylopore diameters of *M. complanata* were larger ($p < 0.0001$) than those of *M. alcicornis* morphotypes. The dactylopores of *M. alcicornis* encrusted were larger than those of the branched morphotype (Figure 2).

The distances between dactylopores did not differ among *M. complanata* and the two morphotypes of *M. alcicornis* (Additional file 6). The distances from gastropore to the nearest dactylopore and the distance between gastropores were significantly different ($p < 0.001$) among *M. complanata* and the two morphotypes of *M. alcicornis* (Additional file 6). However, the distance between gastropores was highly variable within morphotypes, ranging from 0.26 to 2.95 mm in the encrusted *M. alcicornis*, from 0.33 to 2.88 mm in the branched *M. alcicornis*, and from 0.30 to 2.22 mm in *M. complanata*. Gastropores were more scattered in the encrusted *M. alcicornis* than in the branched *M. alcicornis*, while the colonies of *M. complanata* had the smaller distances between gastropores.

The canonical discriminant function analysis (Wilk's $\lambda = 0.145$, $F = 7.79$, $p < 0.0001$) corroborated the *a priori* assigned groups, with 87.1% ($n = 26$) of the colonies correctly classified (Figure 3). Six colonies were misclassified: one *M. complanata* (McMg3), two branched *M. alcicornis* (Mab1, MabT23), and three encrusted *M. alcicornis* (MaeMg1, MaeLP3, MaeLP5). None of these colonies was misclassified in the phylogenetic analysis (see below, Figure 4). The canonical plot showed three main groups corresponding to the three taxa (Figure 3). *M. complanata* was the most distinct morph. The two morphs of *M. alcicornis* were also differentiated in the plot, although 30% of the colonies (three of each morph) had similar values. A wide range of values for the *M. alcicornis* morphotypes was also observed, illustrating the high trait variability of the colonies. Dactylopore diameter was the variable that better discriminated *M. complanata* from the *M. alcicornis* morphotypes, while distance from gastropore to the nearest dactylopore distinguished the two morphotypes of *M. alcicornis*.

Molecular studies

After editing and end-trimming of the DNA traces, a portion (385 bp) of the mitochondrial COI gene was

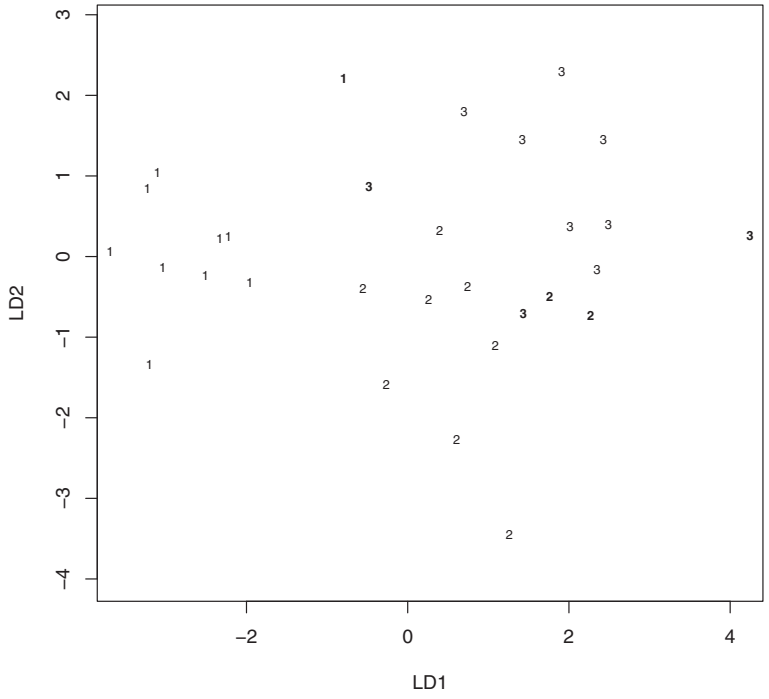

Figure 3 Discriminant function analysis canonical plot based on the morphological traits of the three *Millepora* morphotypes. Multivariate comparisons (fixed effects MANOVA) among morphotypes were significant (Wilk's $\lambda = 0.145$, $F = 7.79$, $p < 0.0001$). Misclassified colonies = 6 (20%), are show in bold. Number 1 represent colonies of *M. complanata* (Mc), no. 2 are colonies of *M. alcicornis* branched (Mab), and no. 3 are colonies of *M. alcicornis* encrusted (Mae). Variables for comparison were as follows: *D*, dactylopore diameter; *G*, gastropore diameter; *G-D*, from gastropore to the nearest dactylopore; *G-G*, distances among gastropores; and *D-D*, distances among dactylopores. In canonical axes 1 and 2, distances among dactylopores and gastropores, and gastropore diameters were the variables with more weight in the discrimination.

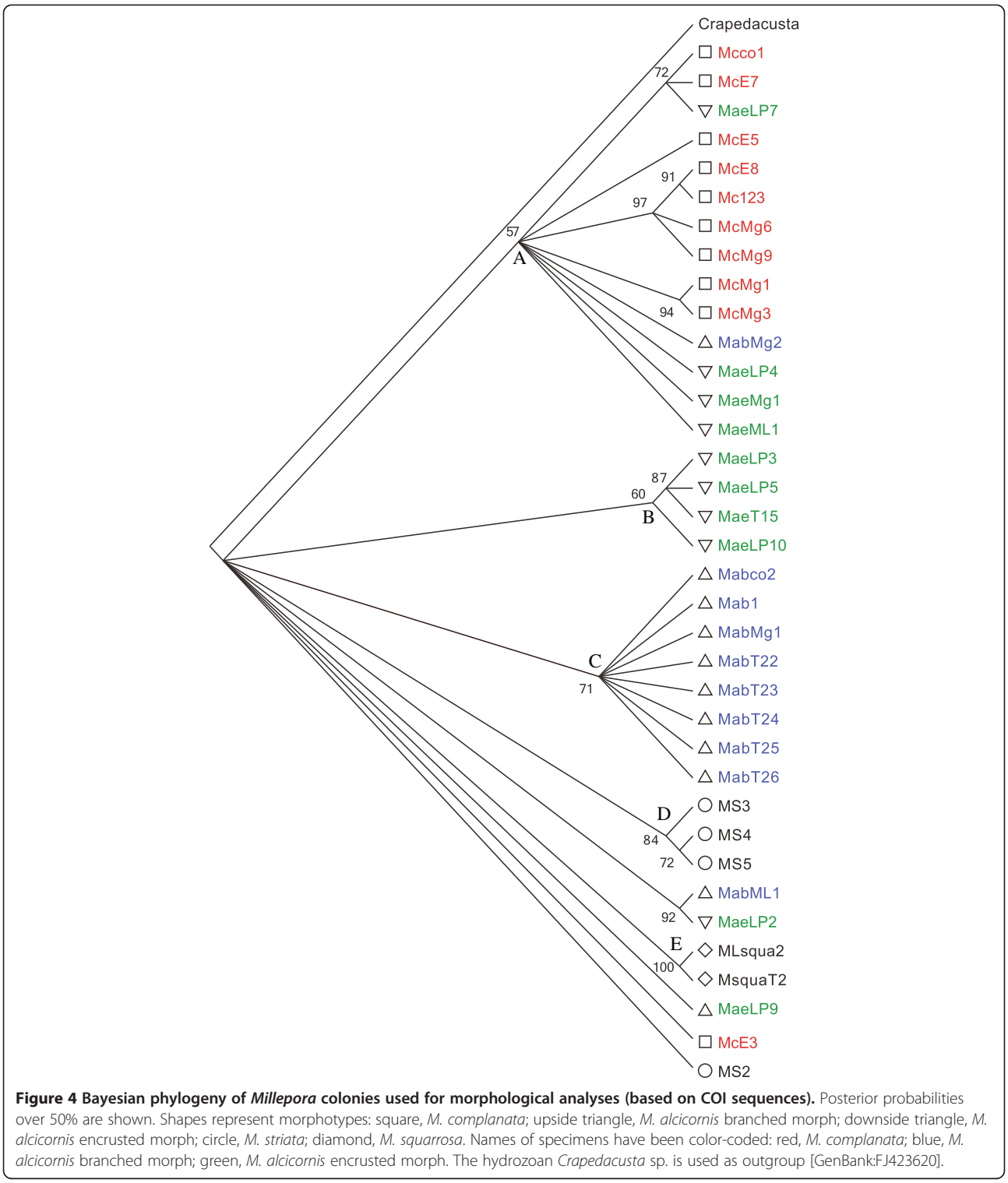

Figure 4 Bayesian phylogeny of *Millepora* colonies used for morphological analyses (based on COI sequences). Posterior probabilities over 50% are shown. Shapes represent morphotypes: square, *M. complanata*; upside triangle, *M. alcicornis* branched morph; downside triangle, *M. alcicornis* encrusted morph; circle, *M. striata*; diamond, *M. squarrosa*. Names of specimens have been color-coded: red, *M. complanata*; blue, *M. alcicornis* branched morph; green, *M. alcicornis* encrusted morph. The hydrozoan *Crapedacusta* sp. is used as outgroup [GenBank:FJ423620].

used for the genetic analyses. Each colony used for the morphological study was sequenced to measure levels of variation in COI. COI sequences of the other Caribbean species were added to the data set, for a total of 36 sequences (10 *M. complanata*, 10 *M. alcicornis* branched, 10 *M. alcicornis* encrusted, 4 *M. striata*, and 2 *M. squarrosa*).

Overall, 25 haplotypes were identified, with 70% of the sequences being unique (Figure 5). Of those haplotypes, six belong to *M. complanata*, five to *M. alcicornis*

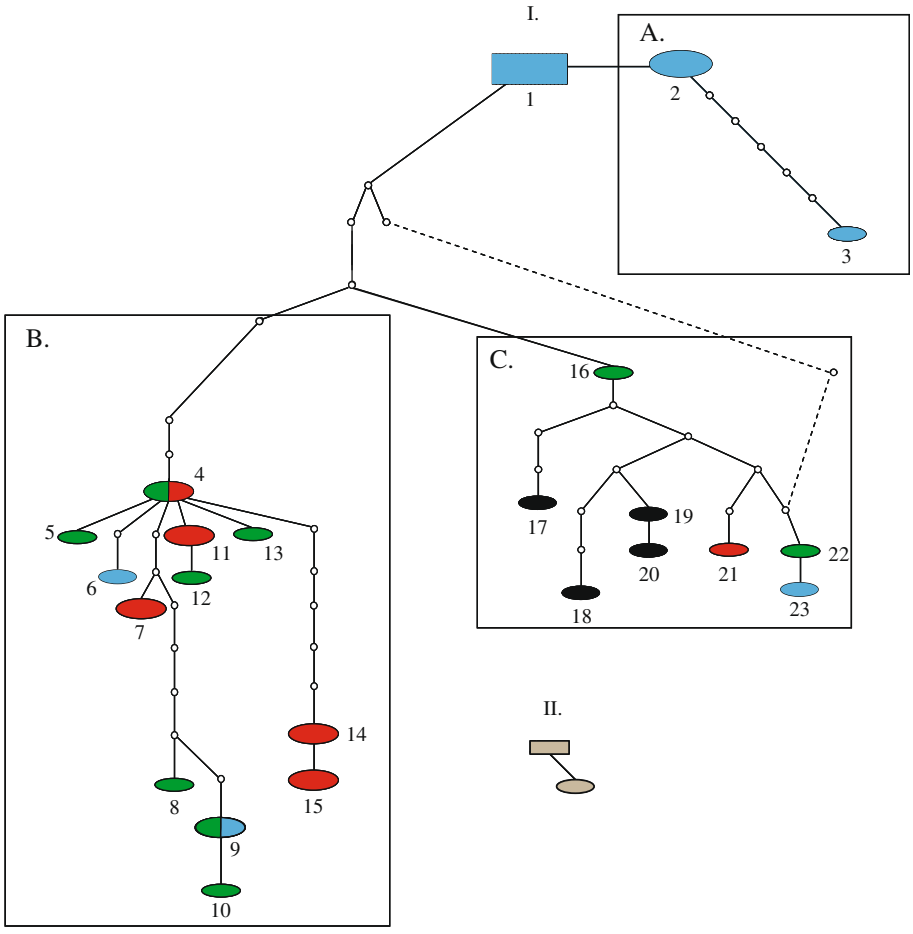

Figure 5 Parsimony haplotype network of the morphotypes based on COI sequences. Patterns represent morphotypes: red ovals, *M. complanata*; blue ovals, *M. alcicornis* branched morph; green ovals, *M. alcicornis* encrusted morph; black, *M. striata*; grey, *M. squarrosa*. Squares represent ancestral haplotypes. Size of the ovals and squares is proportional to the observed number of sequences corresponding to the haplotype. Small circles are non-sampled or extinct haplotypes. Dashed lines represent alternative connections among the haplotypes, suggesting the presence of homoplasy. Haplotype network I contains *M. complanata*, *M. alcicornis* branched morph, *M. alcicornis* encrusted morph, and *M. striata*, where **A**, **B**, and **C** are the main clusters. Haplotype network II is formed by *M. squarrosa*.

branched, nine to *M. alcicornis* encrusted, four to *M. striata*, and two to *M. squarrosa* (Figure 5). Almost all of the most common haplotypes were shared among morphotypes of *M. alcicornis* and *M. complanata*, across the Caribbean. One haplotype (no. 4) was shared among *M. complanata* and the encrusted morph of *M. alcicornis*, and the other (no. 9) was shared by the two morphotypes of *M. alcicornis*. All the *M. striata* and *M. squarrosa* haplotypes were unique and were not shared by any other species. Nucleotide diversity (π) and Watterson's theta (θ_w) were similar for all the morphotypes/species, varying from 0.01 to 0.02 (Additional file 5). Tajima's *D* values suggested that the COI sequences did not deviate significantly from neutrality.

Divergence among *M. alcicornis*, *M. complanata*, and *M. striata* was low, with *M. striata* more genetically different from *M. complanata* than from *M. alcicornis*. Despite the absence of fixed differences among all pairwise comparisons (excluding *M. squarrosa*), *M. striata* and *M. complanata* had the highest average nucleotide difference (approximately 12% in Additional file 7). *M. striata* shared four segregating sites with *M. complanata* and the two morphs of *M. alcicornis*. On average, there were nine nucleotide differences between *M. striata* and both morphs of *M. alcicornis*. *M. complanata* shared seven mutations with *M. alcicornis* branched (average nucleotide difference of approximately 9.2%) and 11 mutations with the encrusted morph of *M. alcicornis* (average nucleotide difference of approximately 8.4%). The two morphotypes of *M. alcicornis* shared 9 segregating sites with an average nucleotide difference of 6.9%. Fifty-five to fifty-nine fixed differences were identified among *M. squarrosa* and the other species (Additional file 7) with an average of 63 nucleotide differences. *M.*

squarrosa was the most distinct species with approximately 25% of sequence divergence from the other two species.

The phylogenetic reconstruction based on Bayesian inference of the COI haplotypes assigned most colonies to three major clades (A, B, and C); *M. squarrosa* (E) and *M. striata* (D) formed separate clades (Figure 4). None of the major clades supported monophyletic divisions based on morphology. Eight colonies of the branched morph of *M. alcicornis* formed one clade (C, Pp = 71); however, two colonies of the branched morph of *M. alcicornis* (MabMg2 and MabML1) were embedded in the other clades. In addition, the recovered clades did not correspond to the classification resulting from the micromorphology analyses. For example, in the species *M. complanata*, the clade containing colonies McE8, McMg6, McMg9, and Mc123 had dactylopores with similar diameters with the exception of colony McMg9 (Figure 4A; Figure six in Ruiz-Ramos (2009)). On the other hand, the clade formed by different morphs of *M. alcicornis* (MabML1 and MaeLP2) had dactylopores with equal diameter.

Five of the six colonies that were misclassified with the DFA (Figure 3) were placed within clades of the same morphology (Figure 4). McMg3 clustered with McMg1 in clade A. Mab1 and MabT23 clustered with the other samples of *M. alcicornis* branched in clade C. MaeLP3 and MaeLP5 were placed in clade B. MaeMg1 was placed in cluster A.

The NJ analysis gave similar results to the BI phylogeny; the same main clusters (now A, D, and E, Additional file 8) were recovered. Results based on ML analysis are not shown because the phylogenetic tree was greatly unresolved.

Caribbean-wide phylogeny

The Caribbean-wide phylogeny was estimated from all *Millepora* colonies used for the morphological measurements and from 342 additional specimens of *M. complanata* and the two morphs of *M. alcicornis* (Figure 6). A total of 178 haplotypes were recovered from the sampled *Millepora* colonies, and 68% of them (121) were unique sequences. Twenty-one haplotypes (Figure 5) were shared among two or more morphotypes or species.

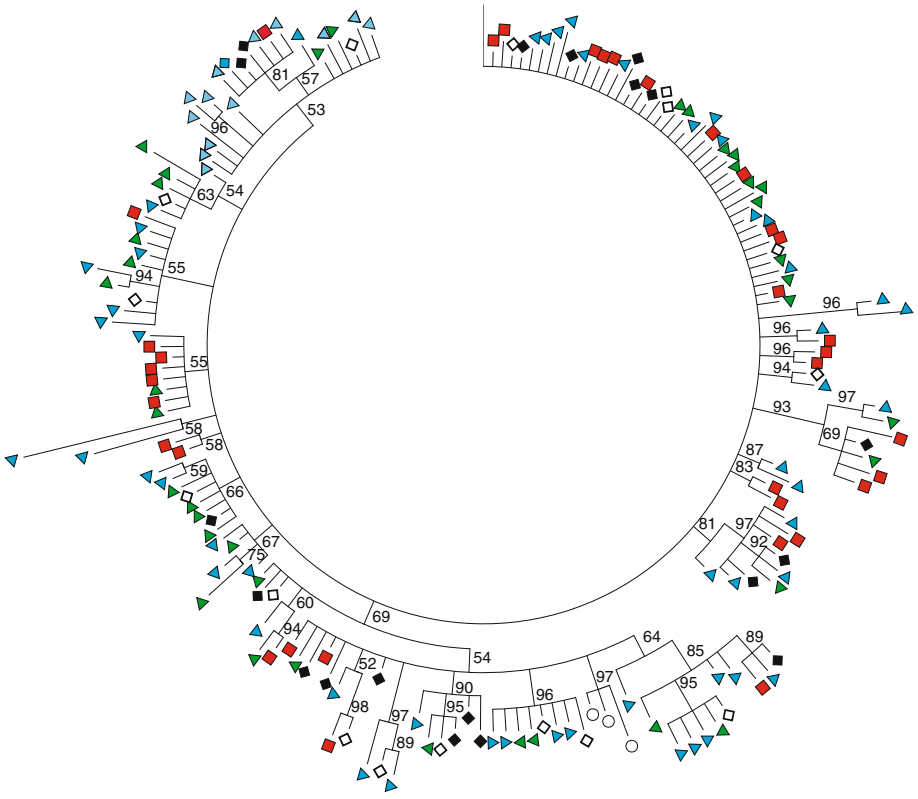

Figure 6 Caribbean-wide phylogeny of *Millepora* based on Bayesian analysis of COI haplotypes. Posterior probabilities over 50% are shown. Shapes represent morphotypes: red square, *M. complanata*; upside blue triangle, *M. alcicornis* branched morph; green downside triangle, *M. alcicornis* encrusted morph; circle, *M. striata*; diamond, haplotypes shared among *M. alcicornis* branched and *M. alcicornis* encrusted; black diamond, haplotypes shared among all the morphotypes. *M. squarrosa* and the hydrozoan *Clytia elsaeoswaldae* [GenBank:DQ064800] was used as the outgroup.

Of the 10 most common haplotypes, five were shared among different morphotypes or species. The specimens of *M. striata* yielded distinct haplotypes; one of them was shared with both *M. alcicornis* and *M. complanata*. Nine main clades were recovered, and as in the previous phylogenies, none of the species were monophyletic (Figure 6). The resulting phylogeny was not concordant with morphologically based species assignments of the colonies, neither with the geographic origin of the samples.

Analysis of molecular variances

An AMOVA test was applied to a portion of the Caribbean data used for the phylogeny analysis (Additional file 9). Samples across the Caribbean from three species (*M. alcicornis*, *M. complanata*, and *M. striata*) were added for a total of seven populations (Mona, La Parguera reef system, Vieques, Panama, Guadeloupe, Grand Cayman, Curaçao). The highest percentage of variation was found within the Caribbean region (89.89%). Significant levels of differentiation were detected among morphotypes within populations ($\Phi_{SC} = 0.067$), among populations ($\Phi_{CT} = 0.037$), and within the Caribbean ($\Phi_{ST} = 0.101$) (Additional file 9). None of the different morphotypes within a population was significantly different from each other (Additional file 10). In contrast, populations were significantly different across the Caribbean, with some of the populations, such as Vieques and Culebra, being only 14 km apart (Additional file 10).

Discussion

The goals of this study were to genetically delineate the four recognized species of Caribbean *Millepora* (*M. alcicornis*, *M. complanata*, *M. striata*, and *M. squarrosa*). The molecular data suggested that three species of *Millepora* exist in the Caribbean: *M. squarrosa*, *M. striata*, and the complex composed of *M. alcicornis* and *M. complanata. M. squarrosa* is highly differentiated at the mitochondrial level from the other species. *M. striata* is closely related to *M. alcicornis* and *M. complanata* since one of its four haplotypes was shared with the species complex. To improve the resolution of the species relationships, we used a multicharacter approach (genetic and morphological data) to discriminate between *M. alcicornis* and *M. complanata*. Analysis of variances of micro-morphological traits showed high variation between *M. complanata*, *M. alcicornis* branched, and *M. alcicornis* encrusted and also among colonies within each morphotype. Dactylopore diameter and distance between gastropores were the only traits in which variation among morphotypes was higher than variation within morphotypes, therefore reliably separating the three morphotypes.

The genetic data was characterized by an unexpected high variability in the COI region; however, the pattern of variation was unrelated to colony shape. The relationships among COI haplotypes recovered by Bayesian inference and neighbor joining were independent of the morphology. Our sampling design had limitations and favored comparisons between *M. alcicornis* and *M. complanata* because they were the dominant milleporids in the sampling locations. *M. squarrosa* populations were decimated from reefs off La Parguera during the bleaching event of 2005, and only a couple colonies were found, so no morphological analyses were undertaken.

These findings question the current status of *Millepora* species in the Caribbean. The limited number of samples showed high morphological variability within and among taxa in the characters measured. However, the genetic results did not support the separation of four species. The presence of intermediate forms and excessive sharing of haplotypes between *M. alcicornis* and *M. complanata* and partially with *M. striata* suggest that these are not discrete species. Whether these morphotypes are ecotypes of one single species, species in the process of divergence or hybrids requires further study. In contrast, *M. squarrosa* stands as a distinct species, and its basal position in the phylogeny suggests that this species is an ancestor of the other Caribbean species. The limited distribution of this species is curious and worth investigating.

For the genetic analysis, *Millepora* colonies were collected from Panama (*M. striata*, *M. complanata*, and the two *M. alcicornis* morphs), Puerto Rico (all except *M. striata*), Curaçao, Guadeloupe, and Grand Cayman (all except *M. striata* and *M. squarrosa*), while the morphometric analysis included *Millepora* colonies from only one location (La Parguera, Puerto Rico). Ideally, a comprehensive study of morphological variation in Caribbean *Millepora* should include several more locations encompassing the known distribution of each species. In very closely related species, the species boundaries may vary according to the geographic origin of the samples as it has been demonstrated in the *Orbicella annularis* (previously *Montastraea annularis*) complex (Fukami et al. 2004). These authors showed that *Orbicella faveolata*, *O. annularis*, and *Orbicella franksi* were genetically and morphologically different in Panama, whereas in the Bahamas, all three species show some degree of genetic and morphological convergence. Subsequently, Levitan et al. (2011) found colonies in Panama that are morphologically similar to *O. franksi* but genetically associated with *O. annularis*. Similarly, we find ambiguous boundaries in the Caribbean *Millepora*; however, we recognize that our sampling regime does not cover the known distribution of *Millepora*.

Previous authors have noted high intraspecific variability in the micro-morphological traits of *Millepora*, despite their usefulness. Boshma (1948) suggested that

colony shape was the only character distinct enough to be useful in the taxonomic classification of the genus. Other studies (e.g., Martínez-Estalella 1982) concluded that the use of skeletal traits was impractical due to their high intraspecific variation. However, de Weerdt (1984) suggested that the size and density of dactylopores could have some taxonomic value when used in combination with colony shape. *M. alcicornis*, *M. complanata*, and intermediate growth forms found in San Salvador, Bahamas were discriminated morphologically on the basis of skeletal micro-structures (Squiers et al. 2011). ITS sequences revealed contradictory results, as pre-assigned specimens of the two Bahamian *Millepora* species were also partitioned into different genetic clades (Squiers et al. 2011). Our results corroborate those of de Weerdt (1984), when only one micro-morphological character was used; high variability among colonies within and across species was evident. Nevertheless, caution must be exercised as the traits were very variable, and some overlapping was observed among colonies of different morphologies. Additionally, some of the five characters used in the analysis may covary, therefore violating the assumption of character independence.

Phenotypic plasticity and the environment

In marine environments, water energy, light availability and sediment transport have been shown to influence the development and survival of the organisms (Yoshioka and Yoshioka 1989, 1991). The different morphologies of *Millepora* have been attributed to the different environmental regimes along the reef structure (de Weerdt 1981; Vago et al. 1994; Kaandorp 1999). Generally, delicate branching types are distributed in calm or deep waters, while blade-like and encrusting morphs are conspicuous in high-energy environments such as the reef crest (Lewis 2006). In the reef crest, *Millepora* is a fast colonizer of the substratum; a bladed morphology may limit the water impact and drag force while attaining more surface area to trap light and plankton. In habitats with limited substrate, a branching morphology facilitates exploitation of the resources in the water column, increasing the surface area for feeding and light exposure (Coates and Jackson 1986; Harper 1986; Todd 2008). Additionally, branches can be advantageous in high-energy environments because fragmentation may help the coral to colonize and monopolize extensive habitats and increase the number of ramets (Jackson 1986; Harper 1986). In the case of *M. alcicornis*, branches may also help to localize, reach, and overgrow weaker space competitors, such as *Plexaura homomalla* (Wahle 1980) and *Gorgonia ventalina*. The overgrowth of *G. ventalina* by *M. alcicornis* may be associated with an increase of the surface area of the hydrozoan colony and, perhaps, a decrease of the energy expended in calcification.

The individual zooids may be affected by the micro-environmental conditions, showing variability in the size of the zooids within a colony. For example, the amount of light received by the zooids in the upper and lower parts of the colonies can be different, and consequently, growth rates of the zooids may also differ. In addition, the types of substratum being covered could modify the shape and size of the zooids in encrusted forms of *Millepora*.

In this study, the morphotypes of *M. complanata*, *M. alcicornis* branched, and *M. alcicornis* encrusted were recovered with the DFA, suggesting a relationship between microskeletal characters and colony shape. Dactylopore diameter and the distance between gastropores were the most diagnostic characters. Diameter of the gastropore also influenced the clustering. Whether the environment influences the morphotypes needs further investigation. Previous transplantation experiments have provided evidence for the ability of *Millepora* to change the colony shape when different environmental conditions are encountered (de Weerdt 1981; Meroz-Fine et al. 2003). However, the presence of different morphotypes occurring side by side in the reef suggests that part of this morphological plasticity is genetically controlled.

Genetic variation

High intraspecific variability was not only observed at the phenotypic level but also at the molecular level; COI sequences showed high levels of polymorphism with 68% of the sequences being unique haplotypes. Despite the high number of haplotypes, sequences were not divergent enough to distinguish among species. *M. squarrosa* was an exception, as it was very different from the other morphs, with an average of 56 fixed mutations (25% difference). In general, genetic differentiation was higher within Caribbean populations than among the different morphotypes. However, pairwise differences were significant for *M. striata* and *M. alcicornis* from Panama, and *M. alcicornis* and *M. complanata* from La Parguera (SW) and Culebra. The lack of genetic differentiation among *M. complanata* and *M. alcicornis* provides evidence for a species complex with some geographical differentiation. Phylogenetic and haplotype network analyses did not recover a monophyletic *M. striata* even though significant, but small pairwise differences were found between *M. striata* and the complex *M. complanata-M. alcicornis*. Therefore, more data is needed to determine whether *M. striata* belongs to the species complex.

In the Red Sea, *Millepora dichotoma* exhibited four recognized morphotypes: delicate branched, blade-like branched, encrusting, and box-work morphs (Vago et al. 1998). Molecular studies (Meroz-Fine et al. 2003) showed that the four morphotypes consisted of two species; each

species was represented by two different morphotypes. Similar results were found for the *Millepora* species occurring in Vietnam (Manchenko et al. 1993). Our study revealed similar patterns in the Caribbean, where the branched (*M. alcicornis*) and bladed (*M. complanata*) morphs were genetically indistinguishable with a neutral molecular marker. The cross-blade *M. striata* gave inconclusive results. The thick box-work *M. squarrosa* was the only distinct species.

Mitochondrial DNA is highly conserved within scleractinian corals (Anthozoa) (Shearer et al. 2002; Hellberg 2006), but for *Millepora* and other members of the Medusozoa (sister group to Anthozoa), the rate of mitochondrial DNA evolution is similar to those found in the Bilateria (Govindarajan et al. 2005; Hellberg 2006). The COI was successfully used to identify sibling species within the Medusozoa (Dawson and Jacobs 2001; Govindarajan et al. 2005). The analyzed portion of the COI gene (385 bp) contained 84 variable sites, of which 75 were parsimony informative, confirming the usefulness of mtDNA in species-level phylogenies in Medusozoa. Therefore, the lack of genetic divergence among *M. alcicornis*, *M. complanata*, and *M. striata* is not a consequence of low levels of mtDNA variation.

None of the mutations were fixed among *M. alcicornis*, *M. complanata*, and *M. striata*, but more than 50 mutations were fixed in *M. squarrosa*. Sequence divergence ranged from 22% to 25% among *M. squarrosa* and the other morphotypes, and from 0% to 3% between *M. alcicornis*, *M. complanata*, and *M. striata*. Values of sequence divergence ≥4% in the mtDNA were previously considered as a threshold for the distinction among species (Dawson and Jacobs 2001; Knowlton and Weigt 1998).

Taxonomical and genetic incongruence of the M. complanata-M. alcicornis complex

The multicharacter approach (e.g., morphological, molecular, behavioral, life history strategy, and reproductive biology) in corals has been shown to successfully differentiate species, which could not be distinguished with one set of characters (Knowlton et al. 1992; van Veghel and Bak 1993; Weil and Knowlton 1994; Levitan et al. 2004). Our two-character approach (morphology and genetics) was inconclusive. The morphological characters agreed with the prevailing taxonomic status of *M. complanata* and *M. alcicornis*; morphological samples from *M. squarrosa* and *M. striata* need to be compared. The genetic data suggest *M.* that *squarrosa* is a distinct species.

Studies in corals highlight the limitation of applying a particular species concept in a group of recently divergent taxa (e.g., *Orbicella*, Pacific *Acropora*) that occupy similar habitats, exhibit phenotypic plasticity, and have no temporal and physical reproductive barriers (i.e., during mass spawning). *Orbicella* species are morphologically distinct, but intermediate morphs can be found (Weil and Knowlton 1994). *Orbicella* spp. are composed of three sibling species (Weil and Knowlton 1994), but evidence for a single species in Curaçao and Florida had been suggested by others (van Veghel and Bak 1993; Medina et al. 1999). Follow-up studies showed that the geographical differences in the complex were due to a latitudinal hybridization gradient through the Western Atlantic (Fukami et al. 2004; Levitan et al. 2011).

The application of a species concept in *Millepora complanata* and *M. alcicornis*, two widely distributed milleporids in the Caribbean, is equally problematic. The biological species concept cannot be tested yet due to lack of studies on the reproduction of *Millepora. M. complanata* and *M. alcicornis* formed paraphyletic clusters in the phylogenetic tree; therefore, the taxa did not meet the species criteria according to the phylogenetic species concept either. Additional support for this assertion is stemming from an independently involved nuclear ribosomal region (ITS), which resulted in clades containing both *Millepora* species and intermediate forms (Squiers et al. 2011; Tepper et al. 2012).

Whether the *Millepora* species are a single evolutionary entity or an example of incipient speciation or reticulate evolution is unknown. The presence of intermediate forms and the paraphyly of *Millepora* species indicate the possibility of reticulate evolution, which is a common phenomenon in corals (Veron 1995; Hatta et al. 1999; van Oppen et al. 2000; Richards et al. 2008). On the other hand, if *M. alcicornis* and *M. complanata* are undergoing speciation, lineage sorting of the COI (this study) and ITS region (Squiers et al. 2011; Tepper et al. 2012) is still incomplete, suggesting recent divergence.

Conclusions

This study suggests that the Caribbean milleporids include two species, *M. squarrosa* and the species complex of *M. alcicornis-M. complanata*. More information is needed to delineate *M. striata*. The morphological analysis of *M. squarrosa* and *M. striata* is recommended. The use of other molecular markers and the integration of reproduction studies along geographical gradients may help to describe the *Millepora* species complex.

Additional files

Additional file 1: List of the samples used for the morphological and genetic analysis. Code name used throughout the study, morphotype, name of reef, zone within the reef, and depth (in meters) in which the colony was found.

Additional file 2: The reef system of La Parguera, southwestern Puerto Rico. All colonies were sampled from the front reef of the

Enrique (17.954°N, 67.051°W), Media Luna (17.938°N, 67.041°W), Las Pelotas (17.956°N, 67.073°W), Turrumote (17.934°N, 67.018°W), and Margarita (17.922°N, 67.097°W) reefs.

Additional file 3: Morphotypes of *Millepora alcicornis*. Left: *M. alcicornis* free growth branching; right: *M. alcicornis* encrusted on gorgonians.

Additional file 4: Measured traits of coralla in *Millepora alcicornis* (MabT22): g, gastropore; d, dactylopore; 1, diameter of gastropore; 2, diameter of dactylopore; 3, distance among dactylopores; 4, distances from gastropore to the nearest dactylopore; 5, distances among gastropores.

Additional file 5: Genetic diversity and summary statistics based on COI sequences. N, number of samples; S, segregating sites; M, mutations; S.S., synonymous sites; N.S., non-synonymous sites; H, number of haplotypes; Hd, haplotype diversity; π, nucleotide diversity; θ_w, Watterson's theta; SD, standard deviation.

Additional file 6: Variability (mean, median, and 5th and 95th percentiles) in the skeletal traits of *Millepora*. The top graph shows variability in distances among dactylopores, the center graph shows variability in distances from gastropore to the nearest dactylopore, and the bottom graph shows variability in distances between gastropores. The solid line is the median, and the dashed line is the mean. The white boxes represent *M. complanata* (Mc), boxes with vertical lines is *M. alcicornis* branched (Mab), and boxes with diagonal lines is *M. alcicornis* encrusted (Mae). Letters above the boxes are values of Tukey's test; significant differences among positions are denoted by different letters.

Additional file 7: Estimates of COI divergence among the species and morphotypes of *Millepora*. *M. striata*, *M. squarrosa*, *M. complanata*, and *M. alcicornis* (b is the branching morphotype, e is the encrusted morphotype).

Additional file 8: Neighbor joining phylogeny of *Millepora* colonies used for morphological analyses (based on COI sequences). Posterior probabilities over 50% are shown. Shapes represent morphotypes: square, *M. complanata*; upside triangle, *M. alcicornis* branched morph; downside triangle, *M. alcicornis* encrusted morph; circle, *M. striata*; diamond, *M. squarrosa*. Names of specimens have been color-coded as in Figure 4. The hydrozoan *Crapedacusta* spp. is used as outgroup [GenBank: FJ423620].

Additional file 9: Analysis of molecular variances (AMOVA) of *Millepora* species and morphotypes. Comparisons of morphotypes were made within the Caribbean basin, among populations, and among morphotypes within populations. Populations were sampled from Panama, Grand Cayman, Mona, La Parguera reef system, Vieques, Guadeloupe, and Curaçao. Φ_{ST} values were obtained by randomization of 25,000 permutations Bonferroni corrected. *$p < 0.002$, **$p < 0.0001$. Fixation indices $\Phi_{SC} = 0.066$**, $\Phi_{ST} = 0.101$**, $\Phi_{CT} = 0.037$*.

Additional file 10: Pairwise comparisons (Tamura-Nei distance) of *Millepora* populations assigned by morphotypes. Mc, *M. complanata*; Ms, *M. striata*; Mab, *M. alcicornis* branched; Mae, *M. alcicornis* encrusted; Pa, Panama; GC, Grand Cayman; Mo, Mona; SW, La Parguera Reef System; Vi, Vieques; Gd, Guadeloupe. Significant values ($p < 0.01$) are in bold.

Abbreviations

BI: Bayesian inference; COI: cytochrome oxidase I; Mab: *Millepora alcicornis* branched; Mae: *Millepora alcicornis* encrusted; Mc: *Millepora complanata*; MS: *Millepora striata*; PR: Puerto Rico.

Competing interests

The authors declare they have no competing interests.

Authors' contributions

DVRR and NVS conceived and designed the study. DVRR collected and processed the samples, analyzed the data, and drafted the manuscript. NVS collected samples, supervised the study, and helped analyze the data and draft the manuscript. EW collected samples, supervised the morphological analysis, and helped draft the manuscript. All authors read and approved the final manuscript.

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References

Amaral FD, Broadhurst MK, Cairns SD, Schlenz E (2002) Skeletal morphometry of *Millepora* occurring in Brazil, including a previously undescribed species. Proc Biol Soc Wash 115:681–695

Amaral FD, Steiner AQ, Broadhurst MK, Cairns SD (2008) An overview of the shallow-water calcified hydroids from Brazil (Hydrozoa: Cnidaria), including the description of a new species. Zootaxa 1930:56–68

Boshma H (1948) The species problem in *Millepora*. Zool Verhandel 1:3–115

Cairns SD (1999) Species richness of recent Scleractinia. Atoll Res Bull 459:1–46

Clement M, Posada D, Crandall K (2000) TCS: a computer program to estimate gene genealogies. Mol Ecol 9:1657–1660

Coates AG, Jackson JBC (1986) Morphological themes in the evolution of clonal and aclonal marine invertebrates. In: Jackson JBC, Buss LW, Cook RE (eds) Population biology and evolution of clonal organisms. Yale University Press, New Haven, Massachusetts, p 67

Dawson MN, Jacobs DV (2001) Molecular evidence for cryptic species of *Aurelia aurita* (Cnidaria, Scyphozoa). Biol Bull 200:92–96

de Weerdt WH (1981) Transplantation experiments with Caribbean *Millepora* species (Hydrozoa, Coelenterata) including some ecological observations on growth forms. Bijdr Dierkd 51:1–19

de Weerdt WH (1984) Taxonomic characters in Caribbean *Millepora* species (Hydrozoa, Coelenterata). Bijdr Dierkd 54:243–262

de Weerdt WH (1990) Discontinuous distribution of the tropical West Atlantic hydrocoral *Millepora squarrosa*. Beaufortia 41:195–203

Edmunds PJ (1999) The role of colony morphology and substratum inclination in the success of *Millepora alcicornis* on shallow coral reefs. Coral Reefs 18:133–140

Excoffier L, Larval G, Schneider S (2005) Arlequin ver. 3.0: an integrated software package for population genetics data analysis. Evol Bioinform 1:47–50

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131:479–491

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Fenner D (1999) New observations on the stony coral (Scleractinia, Milleporidae, and Stylasteridae) species of Belize (Central America) and Cozumel (Mexico). Bull Mar Sci 64:143–154

Fukami H, Budd AF, Levitan DR, Jara J, Kersanach R, Knowlton N (2004) Geographic differences in species boundaries among members of the *Montastraea annularis* complex based on molecular and morphological markers. Evolution 58:324–337

Glynn PW (1993) Coral reef bleaching: ecological perspectives. Coral Reefs 12:1–17

Govindarajan AF, Halanych KM, Cunningham CW (2005) Mitochondrial evolution and phylogeography in the hydrozoan *Obelia geniculata* (Cnidaria). Mar Biol 146:213–222

Harper JL (1986) Modules, branches and the capture of resources. In: Jackson JBC, Buss LW, Cook RE (eds) Population biology and the evolution of clonal organisms. Yale University Press, New Haven, Massachusetts, p 1

Hatta M, Fukami H, Wang W, Omori M, Shimoike K, Hayashibara T, Ina Y, Sugiyama T (1999) Reproductive and genetic evidence for a reticulate evolutionary history of mass-spawning corals. Mol Biol Evol 16:1607–1613
Hellberg ME (2006) No variation and low synonymous substitution rates in coral mtDNA despite high nuclear variation. BMC Evol Biol 6:24
Jackson JBC (1986) Distribution and ecology of clonal and aclonal benthic invertebrates. In: Jackson JBC, Buss LW, Cook RE (eds) Population biology and evolution of clonal organisms. Yale University Press, New Haven, Massachusetts, pp 297–356
Kaandorp JA (1999) Morphological analysis of growth forms of branching marine sessile organisms along environmental gradients. Mar Biol 134:295–306
Kimura M (1968) Evolutionary rate at the molecular level. Nature 217:624–626
Knowlton N, Weigt A (1998) New dates and new rates for divergence across the Isthmus of Panama. Proc R Soc Lond B 265:2257–2263
Knowlton N, Weil E, Weight LA, Guzmán HM (1992) Sibling species in *Montastraea annularis*, coral bleaching, and the coral climate record. Science 255:330–333
Levitan DR, Fukami H, Jara J, Kline D, McGovern TM, McGhee KE, Swanson CA, Knowlton N (2004) Mechanisms of reproductive isolation among sympatric broadcast-spawning corals of the *Montastraea annularis* species complex. Evolution 58:308–323
Levitan DR, Fogarty ND, Jara J, Lotterhos KE, Knowlton N (2011) Genetic, spatial, and temporal components of precise spawning synchrony in reef building corals of the *Montastraea annularis* species complex. Evolution 65:1254–1270
Lewis JB (1989) The ecology of *Millepora*. Coral Reefs 8:99–107
Lewis JB (1992) Heterotrophy in corals: zooplankton predation by the hydrocoral *Millepora complanata*. Mar Ecol Prog Ser 90:251–256
Lewis JB (2006) The biology and ecology of the hydrocoral *Millepora* on coral reefs. Adv Mar Biol 50:1–55
Maddison WP, Maddison DR (2000) Macclade 4: analysis of phylogeny and character evolution. Sinauer Associates Inc, Sunderland, Massachusetts
Manchenko GP, Moschenko AV, Odintsov VS (1993) Biochemical genetics and systematics of *Millepora* (Coelenterata: Hydrozoa) from the shore of south Vietnam. Biochem Syst Ecol 21:729–735
Marshall PA, Baird AH (2000) Bleaching of corals on the Great Barrier Reef: differential susceptibilities among taxa. Coral Reefs 19:155–163
Martínez-Estalella N (1982) Sistemática del género *Millepora* (Hydrozoa: Milleporidae) y datos sobre algunos organismos asociados. Poeyana 246:1–27
Medina M, Weil E, Szmant A (1999) Examination of the *Montastraea annularis* species complex (Cnidaria: Scleractinia) using ITS and COI sequences. Mar Biotech 1:89–97
Meroz-Fine E, Brickner I, Loya Y, Ilan M (2003) The hydrozoan coral *Millepora dichotoma*: speciation or phenotypic plasticity? Mar Biol 143:1175–1183
Paulay G, Benayahu Y (1999) Patterns and consequences of coral bleaching in Micronesia (Majuro and Guam) in 1992–1994. Micronesica 31:109–124
Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. Bioinformatics 14:817–181
Razak TB, Hoeksema BH (2003) The hydrocoral genus *Millepora* (Hydrozoa: Capitata: Milleporidae) in Indonesia. Zool Verhandel 345:313–336
Richards ZT, Oppen MJH, Wallace CC, Willis BL, Miller DJ (2008) Some rare Indo-Pacific coral species are probable hybrids. PLoS ONE 3:1–7
Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574
Rozas J, Sánchez-DelBarrio JC, Messeguer X, Rozas R (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19:2496–2497
Ruiz-Ramos DV (2009) Morphological and genetic variation in the Caribbean species of the hydrocoral genus *Millepora*. University of Puerto Rico, Mayagüez, Dissertation
Shearer TL, Van Oppen MJH, Romano SL, Worheide G (2002) Slow mitochondrial DNA sequence evolution in the Anthozoa (Cnidaria). Mol Ecol 11:2475–2487
Squiers L, Hay C, Gorbach D, Friend D, Black B, Greenstein B, Strychar K, Tepper C (2011) Cryptic speciation: a mismatch between genetics and morphology in *Millepora*. The 13th Symposium on the Natural History of the Bahamas 13:8–19
Swofford DL, PAUP* (2002) Phylogenetic analysis using parsimony*, version 4.0b10. Sinauer Associates Inc, Sunderland, Massachusetts
Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585–595
Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol 10:512–526
Tepper C, Squiers L, Hay C, Gorbach D, Friend D, Black B, Greenstein B, Strychar K (2012) Cryptic species: a mismatch between genetics and morphology in *Millepora*. Mar Sci 2:57–65
Todd PA (2008) Morphological plasticity in scleractinian corals. Biol Rev 83:315–337
Vago R, Shai Y, Ben-Zion M, Dubinsky Z, Achituv Y (1994) Computerized tomography and image analysis: a tool for examining the skeletal characteristics of reef building organisms. Limn Oceanogr 39:448–452
Vago R, Achituv Y, Vaky L, Dubinsky Z, Kizner Z (1998) Colony architecture of *Millepora dichotoma* Forskal. J Exp Mar Biol Ecol 224:225–235
Van Oppen MJH, Willis BL, van Vugt HWJA, Miller DJ (2000) Examination of species boundaries in the *Acropora cervicornis* group (Scleractinian, Cnidaria) using nuclear DNA sequence analyses. Mol Ecol 9:1363–1373
Van Veghel MLJ, Bak RPM (1993) Intraspecific variation of a dominant Caribbean reef building coral, *Montastraea annularis*: genetic, behavioral and morphometric aspects. Mar Ecol Prog Ser 92:255–265
Veron JEN (1995) Corals in space and time: the biogeography and evolution of the Scleractinia. Cornell University Press, Ithaca, New York
Wahle CM (1980) Detection, pursuit, and overgrowth of tropical gorgonians by milleporid hydrocorals: Perseus and Medusa revisited. Science 209:689–691
Weil E, Knowlton N (1994) A multi-character analysis of the Caribbean coral *Montastraea annularis* (Ellis and Solander, 1786) and its two sibling species, *M. faveolata* (Ellis and Solander, 1786) and *M. franksi* (Gregory, 1895). Bull Mar Sci 55:151–175
Yoshioka PM, Yoshioka BB (1989) Effects of water motion, topographic relief and sediment transport on the distribution of shallow-water gorgonian community. Mar Ecol Prog Ser 54:257–264
Yoshioka PM, Yoshioka BB (1991) A comparison of the survivorship and growth of shallow-water gorgonian species of Puerto Rico. Mar Ecol Prog Ser 69:253–260

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