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REPORT

An integrated morpho-molecular approach to delineate species boundaries of *Millepora* from the Red Sea

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Abstract Fire corals of the hydrocoral genus *Millepora* provide an important ecological role as framework builders of coral reefs in the Indo-Pacific and the Atlantic. Recent works have demonstrated the incongruence between molecular data and the traditional taxonomy of *Millepora* spp. based on overall skeleton growth form and pores. In an attempt to establish a reliable and standardized approach for defining species boundaries in *Millepora*, we focused on those from the Red Sea. In this region, three species are currently recognized: the fan-shaped branching *M. dichotoma*, the blade-like *M. platyphylla*, and the massive/encrusting *M. exaesa*. A total of 412 colonies were collected from six localities. Two mitochondrial marker genes (COI and 16S rDNA) were sequenced to obtain phylogeny reconstructions and haplotype networks. Eight morphological traits of pores and the nematocysts of both polyp and eumedusoid stages were measured to determine whether significant morphological differences occur among the three species. Both markers clearly resolved *M. dichotoma*, *M. platyphylla*, and *M. exaesa* as distinct, monophyletic lineages in the Red Sea. Nevertheless, they also revealed deep genetic breaks with Southwestern Indian Ocean populations of the three species. In the Red Sea, the three species were further distinguished based on their pore and nematocyst features. A discriminant analysis revealed dactylopore density, number of dactylopores per gastropore, dactylopore distance, and gastropore diameter as the most informative discriminative characters. The heteronemes, the large and small stenoteles of polyps, and the distribution of mastigophores of eumedusoids also showed significant interspecific differences. An integrated morpho-molecular approach proved to be decisive in defining species boundaries of *Millepora* supported by a combination of pore and nematocyst characters, which may be phylogenetically informative.

Keywords Fire corals · Phylogeny · Pore · Nematocyst · Eumedusoid

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Introduction

Species of the hydrocoral genus *Millepora* are popularly known as fire corals (Pourtalès 1877). They are colonial organisms building persistent calcareous skeletons and as such play an important ecological role as framework builders of coral reefs (Lewis 2006). These hydrocorals are

among the most relevant reef builders in shallow-water tropical seas, second only to scleractinians (Lewis 1989; Edmunds 1999; Smith et al. 2014). *Millepora* occurs in tropical and subtropical coral reefs of both the Atlantic and the Indo-Pacific, and its depth distribution is restricted from less than 1 m to about 50 m deep because of the obligate symbiosis with zooxanthellae of the genus *Symbiodinium* (Boschma 1948a, b, 1956; Cairns et al. 1999).

Despite its wide geographical distribution and relevant ecological role, *Millepora* has scarcely been investigated in coral reef studies and, in particular, its taxonomy and systematics are controversial and much debated (Hickson 1898, 1809; Crossland 1948; Boschma 1948a, 1966; Moshchenko 1992, 1997; Razak and Hoeksema 2003; de Souza et al. 2017). Fire corals have been historically identified based on morphological features such as colony growth form, skeletal pores, and ampullae (Klunzinger 1879; Quelch 1884; Hickson 1898; Boschma 1948b, 1949, 1956). Nevertheless, all these morphological characters are intraspecifically variable and even in a single specimen as a result of ecophenotypic variation (de Weerdt 1981, 1984; Boschma 1948a; Moshchenko 1994, 1995a, 1996a; Amaral et al. 1997; Dubé et al. 2017a, b). Although growth form has been traditionally used as the main diagnostic character for species identification (Forskål 1775; Ehrenberg 1834; Duchassaing and Michelotti 1860; Klunzinger 1879; Hickson 1898; Crossland 1941; Boschma 1948a, b), this feature has been shown to be highly variable. For example, *Millepora platyphylla* Hemprich and Ehrenberg, 1834, in Vietnam displayed distinct morphotypes associated with different reef zones and the occurrence of different colony growth forms was hypothesized to be related to factors such as radiation availability, water movement, and settling suspended matter (Moshchenko 1995a). A complicating factor herein is that juvenile *Millepora* corals usually start with an encrusting growth form (see, e.g., Fig. 2g, Hoeksema et al. 2017: Fig. 1), which makes it difficult to distinguish species at early stage. Similarly, pore characters are known to noticeably vary in response to the rapidity of growth and the amount of light on the skeleton surface and some taxonomists have suggested to discard their use in *Millepora* taxonomy (Hickson 1898, 1809; Boschma 1948a, b). However, Moshchenko (1994, 1995b, 1996b) proposed a quantitative approach for the analysis of pore structures and was able to distinguish *M. platyphylla* from the branching *Millepora* species of the Indian Ocean. As a consequence of this morphological variation, traditional morphology-based species identification since the first valid description of a fire coral by Linnaeus (1758) generated more than 50 nominal species of *Millepora*, nine of which are now considered valid in the Indo-Pacific and six

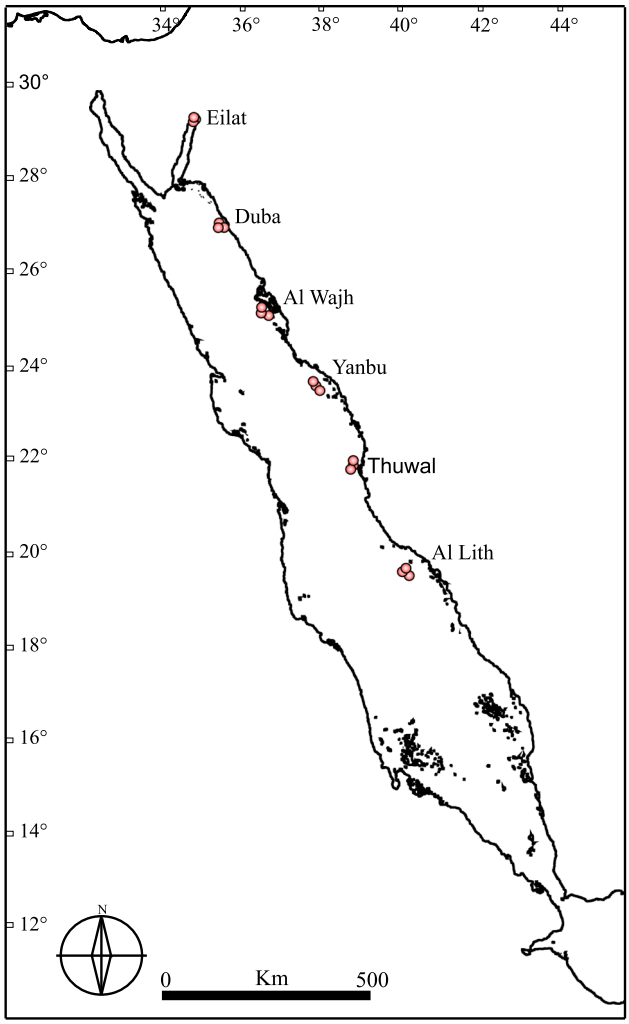


Fig. 1 Map of *Millepora* sampling locations along the Red Sea

in the Atlantic (Razak and Hoeksema 2003; Amaral et al. 2008; de Souza et al. 2017).

Recent outcomes of molecular phylogenetic analyses and DNA taxonomy have provided advantages for the understanding of *Millepora* systematics and connectivity (López et al. 2015; Hoeksema et al. 2017; de Souza et al. 2017; Dubé et al. 2017c). The use of genetics has elucidated the identification and the geographic origin of fire corals in some isolated and remote islands of the central and eastern Atlantic Ocean, including, for example, the Canaries, Cape Verde Islands, and Ascension Island (López et al. 2015; Hoeksema et al. 2017). Moreover, the three endemic species of the Brazilian province, i.e., *Millepora braziliensis* Verrill, 1868, *Millepora nitida* Verrill, 1868, and *Millepora laboreli* Amaral, 2008, were successfully resolved based on molecular data although the traditional morphological characters of pores were not sufficient to discriminate these taxa (de Souza et al. 2017). Nevertheless, other studies have shown discordances

between morphology-based taxonomy and genetics suggesting the need to combine new molecular and morphological data for the definition of species boundaries (Meroz-Fine et al. 2003; Ruiz-Ramos et al. 2014; Takama et al. 2018). For example, Ruiz-Ramos et al. (2014) genetically characterized four Caribbean *Millepora* representatives, i.e., *Millepora alcicornis* Linnaeus, 1758, *Millepora complanata* Lamarck, 1816, *Millepora squarrosa* Lamarck, 1816, and *Millepora striata* Duchassaing and Michelotti, 1864, and recovered the latter two species as distinct molecular lineages while the others form an unresolved species complex. Furthermore, a recent study from Japan demonstrated that the three branching species *Millepora dichotoma* Forskål, 1775, *Millepora intricata* Milne Edwards, 1860, and *Millepora tenera* Boschma, 1949, were recovered in a single molecular clade while a previously unknown lineage of an encrusting *Millepora* form was discovered, which overgrows living scleractinian corals (Takama et al. 2018).

Fire corals are abundant and common throughout the entire Red Sea, where they represent an ecologically important group in shallow-water reef habitats. Three species are reported to occur in the Red Sea (Boschma 1948a, b), i.e., *Millepora dichotoma*, *Millepora platyphylla*, and *Millepora exaesa* Forskål, 1775. The reef crest of the Red Sea reef systems is usually dominated by conspicuous colonies of *M. dichotoma* and *M. platyphylla* in addition to scleractinians (Loya 1972, 1976; Benayahu and Loya 1977; Perkol-Finkel and Benayahu 2004). Consequently, Loya and Slobodkin (1971) introduced the concept of "*Millepora* zone" to identify the reef portion at depths of 0.2–3 m. Because of this abundance, early taxonomists of the Red Sea coral fauna focused their attention on fire corals and provided detailed descriptions of their morphological characters. Notably, the three aforementioned species were described from the Red Sea (Forskål 1775; Ehrenberg 1834; Klunzinger 1879; Crossland 1941). The morphological variation and the colony development of both *M. dichotoma* and *M. exaesa* from the northern tip of the Red Sea (the Gulf of Eilat and Aqaba) were examined and described by Vago et al. (1998) and Meroz-Fine et al. (2003). Although these authors considered *M. exaesa* as an ecomorph of *M. dichotoma*, they found significant differences in molecular data (rDNA internal transcribed spacers), nematocyst capsule size, and colony growth form. Hence, they suggested that the two morphs represent two distinct entities (Meroz-Fine et al. 2003). Nevertheless, these works did not include *M. platyphylla* and evaluated fire corals only from the northern Gulf of Eilat, an isolated part of the Red Sea (Klausewitz 1989; Wolf-Vecht et al. 1992; DiBattista et al. 2016a, b).

With the aim of establishing a reliable and standardized approach for species boundaries definition in *Millepora*, this study uses a combination of molecular and morphological datasets in order to address the degree of concordance between genetic and morphological variation of fire corals from the Red Sea. Two mitochondrial genes and two distinct sets of morphological characters were investigated, and their efficacy to detect species boundaries are discussed in light of our results.

Materials and methods

Sample collection

A total of 412 specimens of *Millepora* were collected while SCUBA diving between 1 and 20 m depth at six localities along the Red Sea (Fig. 1), consisting of 152 colonies of *M. dichotoma*, 128 of *M. platyphylla*, and 132 of *M. exaesa* (Table S1). Specimens were identified to species level following original descriptions (Forskål 1775; Ehrenberg 1834) and the taxonomic revision of Indonesian *Millepora* by Razak and Hoeksema (2003). The two main diagnostic morphological characters used for identification were the colony growth form and the cyclosystem arrangement. During the sampling process, we included all possible morphotypes per species (Fig. 2). To minimize the likelihood of sampling clones, colonies ascribed to the same species were collected at a minimum distance of 5 m (following Ruiz-Ramos et al. 2014). Each coral specimen was photographed underwater (Fig. 2), collected with hammer and chisel, tagged, and approximately 2 cm^3 of the collected colony was sampled and fixed in 95% ethanol. The remaining material was immersed in sodium hypochlorite for 48 h to remove all tissues, rinsed in freshwater, and air-dried for identification and microscope observations. Specimens were deposited at King Abdullah University of Science and Technology (KAUST, Thuwal, Saudi Arabia) and at Steinhardt Museum of Natural History, Tel Aviv University (Tel Aviv, Israel).

DNA extraction, amplification, and sequencing

DNA extractions were carried out with the DNeasy Blood and Tissue kit (Qiagen Inc., Hilden, Germany). A portion of two mitochondrial genes, the cytochrome *c* oxidase subunit I (COI) and the large rRNA subunit (16S rDNA), were obtained from all the collected samples. PCR amplifications of COI were performed using the previously published primers COIF and COIR (Schweinsberg et al. 2016) and the protocol from López et al. (2015), while 16S rDNA was amplified using the primers SHA and SHB (Cunningham and Buss 1993) and the protocol from de Souza et al. (2017). All PCR products were purified with Illustra ExoStar (GE Healthcare, Buckinghamshire, UK)

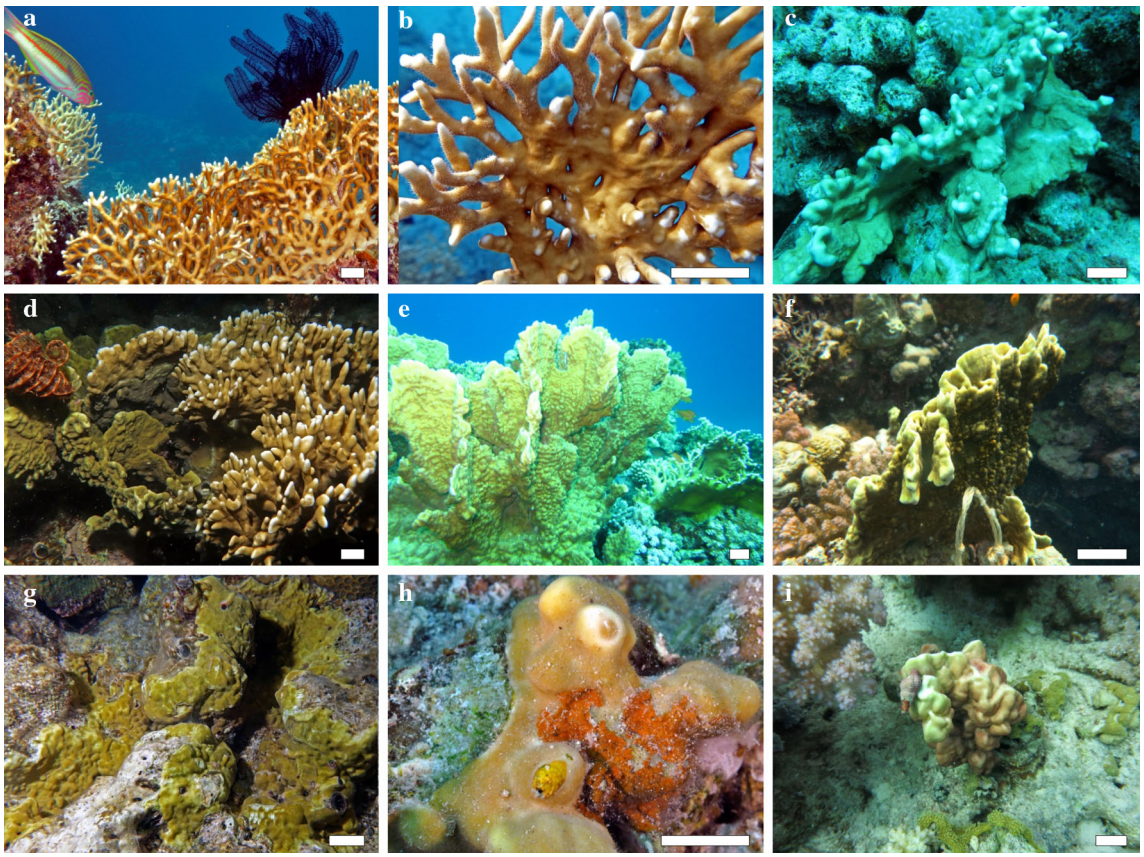


Fig. 2 In situ photographs of the three *Millepora* species occurring in the Red Sea. **a–c** *M. dichotoma*; **d–f** *M. platyphylla*; **g–i** *M. exaesa.* Scale bars: 5 cm

and directly sequenced in forward and reverse directions with the described primers using an ABI 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). Sequences obtained in this study were deposited at GenBank, and accession numbers are listed in Table S1 (MH824796-MH825619).

Genetic data analyses

Chromatograms of the forward and reverse DNA strands were assembled and edited using Sequencher 5.3 (Gene Codes Corp., Ann Arbor, MI, USA). Sequences were aligned with MAFFT 7.397 (Katoh and Standley 2013) and the iterative refinement method E-INS-i. General statistics concerning the obtained sequences and the variability of the two markers were calculated with DnaSP 6.11.1 (Librado and Rozas 2009). Intra- and interspecific genetic distances were calculated as *p*-distance based on the separated COI and 16S rDNA datasets using DnaSP 6.11.1, and variance was estimated with 1000 bootstrap replicates (Tables 1, 2).

In order to define species boundaries of the Red Sea *Millepora*, a median-joining network (Bandelt et al. 1999) for each mitochondrial gene was constructed including exclusively the newly obtained sequences from the Red Sea using Network 5.0 (http://www.fluxus-technology.com). The median-joining method uses a maximum parsimony approach to search for all the shortest phylogenetic trees of given dataset (Bandelt et al. 1999).

All the available COI and 16S rDNA sequences of *Millepora* were downloaded from GenBank and included in our phylogenetic analyses. Because these sequences were derived from different sources and include several species from different localities, we performed separate phylogeny reconstructions for each mitochondrial locus. *Solanderia secunda* was selected as the outgroup for both phylogenetic analyses (Nawrocki et al. 2010; Maggioni et al. 2017a, 2018). Two phylogenetic tree optimality criteria were employed, namely Bayesian inference (BI) using MrBayes (Ronquist et al. 2012) and maximum likelihood (ML) using RAxML (Stamatakis 2014). Both BI and ML analyses were run on the CIPRES server (Miller et al. 2010). Prior to the phylogenetic analyses, we determined the best partition scheme and nucleotide substitution models using PartitionFinder 1.1.1 (Lanfear et al. 2012). We used unlinked branch lengths, the greedy search

Table 1 Pairwise comparisons of genetic distance values (*p* distance) within and between *Millepora* species based on COI

	M. dichotoma Red Sea	*M. dichotoma* Pacific Ocean	*M. platyphylla* Red Sea	*M. platyphylla* Pacific Ocean	*M. exaesa* Red Sea	*M. striata*	*M. alcicornis*	*M. complanata*	*M. squarrosa*
M. dichotoma Red Sea	0.84 ± 0.27	1.22	1.32	1.35	1.86	1.79	1.75	1.80	1.82
M. dichotoma Pacific Ocean	**9.17**	1.13 ± 0.4	1.35	1.33	1.89	1.70	1.67	1.69	1.79
M. platyphylla Red Sea	**9.08**	**9.76**	0.2 ± 0.04	1.34	1.87	1.80	1.78	1.75	1.94
M. platyphylla Pacific Ocean	**10.61**	**10.39**	**10.78**	3.12 ± 0.68	1.93	1.70	1.69	1.71	1.83
M. exaesa Red Sea	**18.11**	**19.48**	**18.77**	**0.19**	0.38 ± 0.16	1.75	1.76	1.76	1.96
M. striata	**16.83**	**17.53**	**17.95**	**0.17**	**16.93**	0.26 ± 0.25	0.42	0.85	1.73
M. alcicornis	**16.81**	**16.85**	**0.18**	**0.18**	**17.10**	**1.15**	0.63 ± 0.27	0.82	1.73
M. complanata	**17.01**	**16.75**	**16.57**	**17.43**	**17.58**	**4.03**	**3.90**	0.52 ± 0.21	1.80
M. squarrosa	**17.00**	**16.95**	**19.87**	**16.67**	**19.92**	**16.36**	**16.45**	**17.53**	0.26 ± 0.24

Interspecific pairwise comparisons of genetic distance are in bold, intraspecific genetic (± standard deviations) distances are indicated along the diagonal, and standard deviations are reported on the upper right-hand portions for each set of comparisons

algorithm for nucleotide sequence, and considered four partitions: 16S rDNA and the three codon positions of COI. Partitioning scheme comparison was performed using the corrected Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC), testing the influence on the tree topology. PartitionFinder 1.1.1 selected for RAxML the evolutionary model GTR+G for all the four partitions and for MrBayes the models of evolution HKY+G for 16S rDNA, K80+G for COI_pos1, F81 for COI_pos2, and HKY+G for COI_pos3. BI runs were performed using four Markov Chain Monte Carlo (MCMC) chains for 10 million generations, saving a tree every 1000th generation. The tree searches were stopped when all parameters reached the stationarity for effective sampling size and unimodal posterior distribution using Tracer 1.6 (Rambaut et al. 2014). The first 25% trees sampled were discarded as burn-in following indications by Tracer. ML topology was obtained under the default parameters shown on the CIPRES server with a multiparametric bootstrap analyses composed of 1000 bootstrap replicates.

Morphological examination

Two sets of morphological features were analyzed in this study, i.e., skeletal pores and polyp and eumedusoid nematocysts. For the examination of skeletal pores, a total of eight characters previously used for taxonomic studies of *Millepora* were selected (Boschma 1948a; de Weerdt 1984; de Weerdt and Glynn 1991; Moshchenko 1995b, 1996b; Razak and Hoeksema 2003) (Fig. 3): (1) gastropore diameter, (2) dactylopore diameter, (3) distance between gastropores, (4) distance between dactylopores, (5) distance from gastropore to nearest dactylopore, (6) number of dactylopores per gastropore, (7) gastropore density, and (8) dactylopore density. Because *M. exaesa* is characterized by absent or limited cyclosystem arrangement, for the analyses we selected only colonies of this species showing a limited but recognizable cyclosytem arrangement. A total of ten colonies selected from those that were genetically characterized were analyzed for each of the three Red Sea *Millepora* species, and a total of ten measurements per colony were taken for each single trait (Table S2). Photographs of the skeleton surface were obtained at × 50 magnification with a Leica IC80 HD microscope and its integrated stand-alone digital camera. On the basis of the digital images with a reference scale, the eight skeletal variables were measured using ImageJ 1.49 (Rasband 1997).

For the examination of polyp nematocysts, five colonies of each *Millepora* species chosen from the ones that were molecularly investigated were decalcified using 10% formic acid for approximately 24 h. Soft tissues were then washed in seawater, observed with a Nikon Eclipse E600

Table 2 Pairwise comparisons of genetic distance values (p distance) within and between *Millepora* species based on 16S rDNA

	M. dichotoma Red Sea	*M. dichotoma* Pacific Ocean	*M. tenera*	*M. platyphylla* Indo-Pacific	*M. platyphylla* Red Sea	*M. alcicornis*	*M. braziliensis*	*M. laboreli*	*M. nitida*	*M. exaesa* Red Sea	*M. exaesa* Indian Ocean
M. dichotoma Red Sea	0.33 ± 0.08	0.81	0.83	0.86	0.97	1.52	1.59	1.66	1.70	1.44	1.52
M. dichotoma Pacific Ocean	**4.30**	0.38 ± 0.20	0.38	0.82	0.88	1.47	1.50	1.49	1.61	1.34	1.44
M. tenera	**4.90**	**1.74**	2.09 ± 0.73	0.82	0.97	1.46	1.53	1.55	1.65	1.46	1.50
M. platyphylla Indo-Pacific	**4.42**	**4.27**	**4.88**	0.27 ± 0.13	0.92	1.45	1.39	1.47	1.54	1.48	1.47
M. platyphylla Red Sea	**5.76**	**5.62**	**6.41**	**5.40**	0.14 ± 0.05	1.55	1.56	1.60	1.70	1.45	1.52
M. alcicornis	**12.13**	**13.21**	**12.98**	**11.91**	**13.13**	0.93 ± 0.25	1.25	1.19	1.34	1.69	1.71
M. braziliensis	**15.69**	**15.52**	**15.49**	**14.43**	**15.54**	**12.86**	1.12 ± 0.31	0.63	0.83	1.64	1.50
M. laboreli	**16.69**	**16.03**	**16.35**	**15.54**	**16.17**	**12.98**	**3.28**	0 ± 0	0.87	1.65	1.55
M. nitida	**16.99**	**17.06**	**17.29**	**16.45**	**16.81**	**14.51**	**5.16**	**4.80**	0.77 ± 0.27	1.68	1.56
M. exaesa Red Sea	**13.67**	**14.72**	**15.51**	**14.65**	**14.27**	**15.16**	**16.40**	**17.52**	**18.21**	0.1 6 ± 0.03	1.01
M. exaesa Indian Ocean	**13.28**	**14.01**	**14.16**	**13.69**	**13.70**	**14.40**	**15.36**	**16.73**	**17.70**	**5.14**	n.c.

Interspecific pairwise comparisons of genetic distance are in bold, intraspecific genetic (± standard deviations) distances are indicated along the diagonal, and standard deviations are reported on the upper right-hand portions for each set of comparisons. n.c. = not calculable

compound microscope, photographed with its integrated camera, and measured using the NIS-Elements Viewer 4.30. For the examination of eumedusoid nematocysts, newly released eumedusoids were collected and anesthetized with menthol crystals. Thereafter, nematocysts were observed with the compound microscope in order to assess their distribution and they were measured. Nematocysts were identified according to Bouillon et al. (2006), and 30–90 undischarged capsules were measured for each type, life stage, and species. A total of six nematocyst features were analyzed, namely (1) polyp large stenotele, (2) polyp small stenotele, (3) polyp macrobasic apotrichous eurytele, (4) polyp macrobasic apotrichous mastigophore, (5) eumedusoid microbasic mastigophore, and (6) eumedusoid stenotele. For each feature, four measurements were obtained, namely length, width, area $\left(\frac{\text{length}}{2} \times \frac{\text{width}}{2} \times \pi\right)$, and ratio $\left(\frac{\text{length}}{\text{width}}\right)$, for a total of 24 analyzed characters. Additionally, polyp heteronemes (euryteles and mastigophores) were discharged adding a drop of sodium hypochlorite to the tissues, and the length of the exploded shaft was measured (Table S3).

Statistical analyses

Morphological variables were log + 1 transformed when the normality distribution was violated, and after addressing assumptions, a subset of the eight pore characters (all but gastropore density) was used in a multivariate analysis of variance (MANOVA) to test for differences in pore characters among *M. dichotoma*, *M. exaesa*, and *M. platyphylla* from the Red Sea. Hereafter, a multiple univariate analysis of variance (ANOVA) was run to determine which characters showed differences among the three investigated species. Wilks' lambda criterion was used to test for group differences in the MANOVA. A discriminant function analysis (DFA) was used to examine the pore characters in discriminating individuals among species and to investigate whether the measured pore characters could be used to classify samples into their original group. A classification with cross-validation was carried out to assign individuals to their original group, and the scatterplots of the first two discriminant scores were drawn to depict the species distribution on the graph.

Furthermore, the nematocyst measurements were investigated in order to detect statistically significant differences among the three investigated species of fire corals. In particular, for the large and small stenoteles, which are found in both the polyp and the eumedusoid stages, and for the two types of heteronemes, i.e., euryteles and mastigophores, which are found only in the polyp stage, the following four measurements were taken into account: length, width, area, and ratio. The measurements were taken by moving in the same direction across the slide in order to measure each nematocyst only once within each specimen in order to avoid pseudoreplication. Statistical differences between the nematocysts of the three fire coral species were tested using the Kruskal–Wallis test when we violated the assumption of normality, and ANOVA when the normality distribution and the homoscedasticity were satisfied.

All the statistical analyses were carried out in SPSS 25 (SPSS, Chicago, IL, USA). All data report mean ± dev. St. otherwise stated.

Results

Species identification

Based on the colony growth form and the cyclosystem arrangement, the sampled specimens were grouped in three species: *M. dichotoma* showing fan-shaped branching growth form with distinct cyclosystems (Figs. 2a–c, 3b), *M. platyphylla* showing a blade-like growth form with distinct cyclosystems on a flat surface (Figs. 2d–f, 3c), and *M. exaesa* showing massive or sub-massive growth form with absent or limited cyclosystem arrangement (Figs. 2g–i, 3d).

Molecular results

COI and 16S rDNA sequences were obtained from all 412 collected fire coral colonies (Table S1). The COI dataset including exclusively our Red Sea *Millepora* samples resulted in 426 bp with 104 variables nucleotides and identified 33 haplotypes. The 16S rDNA alignment included 597 positions with 113 variable nucleotides for a total of 37 haplotypes. Both mitochondrial networks clearly showed the presence of three main clusters of haplotypes (Fig. 4a, b, Fig. S1) corresponding to the three groups of specimens assigned to *M. dichotoma*, *M. platyphylla*, or *M. exaesa* based on growth colony form and cyclosystem arrangement. Individuals ascribed to the same species were very closely related, being separated by a total number of mutations ranging from one to seven. The three species groups were genetically isolated with no shared haplotypes and were distinguished by a high number of mutations, from 28 (M. *platyphylla*—*M. dichotoma* based on 16S rDNA) to 76 (*M. platyphylla*–*M. exaesa* based on COI and *M. dichotoma*–*M. exaesa* based on 16S rDNA).

The two single-gene trees are shown in Fig. 4c–d. In both phylogeny reconstructions, BI and ML analyses were largely congruent with no topology conflicts and node support values were high across the in-group. COI and 16S rDNA yielded similar topologies with well-supported clades although some sister relationships among the

recovered molecular lineages varied between the two phylogenetic trees. Both phylogenetic trees recovered *Millepora* as composed of two main lineages, the Atlantic cluster and the Indo-Pacific one. Within the latter, all the Red Sea samples clustered in three distinct and highly supported molecular clades matching morphological differences based on traditional taxonomy. In fact, clade I includes *M. dichotoma* (COI Bayesian posterior probabilities $PP_{BI} = 1$, ML bootstrap $B_{ML} = 99$; 16S rDNA $PP_{BI} = 1$ and $B_{ML} = 100$), clade II is composed of *M. platyphylla* (COI $PP_{BI} = 1$ and $B_{ML} = 100$; 16S rDNA $PP_{BI} = 1$ and $B_{ML} = 82$), and clade III grouped *M. exaesa* (COI $PP_{BI} = 1$ and $B_{ML} = 100$; 16S rDNA $PP_{BI} = 1$ and $B_{ML} = 93$). The three species from the Red Sea exhibited very high interspecific genetic distances and extremely low intraspecific values (< 1) under both mitochondrial loci (Tables 1, 2). Interspecific genetic distances ranged from 9.08 ± 1.32% (mean ± standard deviation) (*M. dichotoma–M. platyphylla*) to 18.77 ± 1.32% (*M. platyphylla–M. exaesa*) based on COI and between 5.76 ± 0.97% (*M. dichotoma–M. platyphylla*) and 13.67 ± 1.44% (*M. dichotoma–M. exaesa*) based on 16S rDNA. Notably, these three molecular lineages include exclusively our newly obtained sequences from the Red Sea. The sequences downloaded from GenBank of conspecific deriving from other localities of the Southwestern Indo-Pacific did not cluster with any Red Sea sequence and constituted additional and distinct molecular clades. As a result of this, *Millepora dichotoma* and *M. platyphylla* were recovered as polyphyletic taxa while *M. exaesa* was recovered as monophyletic but composed of two divergent and distinct clades. The COI phylogeny reconstruction recovered clade IVa including *M. platyphylla* from French Polynesia (Leray et al. 2013; Schweinsberg et al. 2016) and clade Va composed of *M. dichotoma* from Australia (Schweinsberg et al. 2016). The phylogenetic tree based on 16S rDNA resolved clade IVb with *M. platyphylla* from Réunion Island and Northern Mariana Islands (de Souza et al. 2017), clade Vb consisted

Fig. 4 **a** COI and **b** 16S rDNA most parsimonious median-joining networks of the three Red Sea *Millepora* species. Each circle represents a unique haplotype, and its size is proportional to its total frequency. Thin branches and black cross-bars represent a single-nucleotide change, thick black bars represent greater than one nucleotide change (as indicated), and small black circles represent missing haplotypes. **c** COI and **d** 16S rDNA phylogeny reconstructions of *Millepora* inferred by Bayesian inference. The clade support values are Bayesian posterior probabilities (≥ 0.7) and maximum likelihood bootstrap replicates (≥ 70). Colors denote each distinct molecular lineage as reported in the figure box

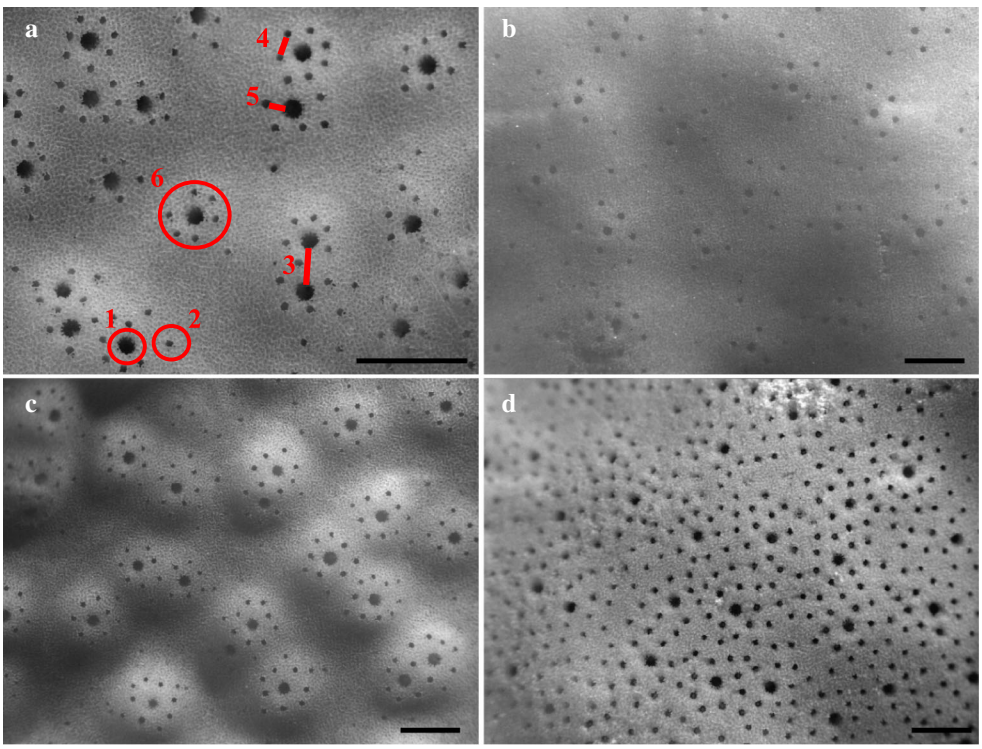
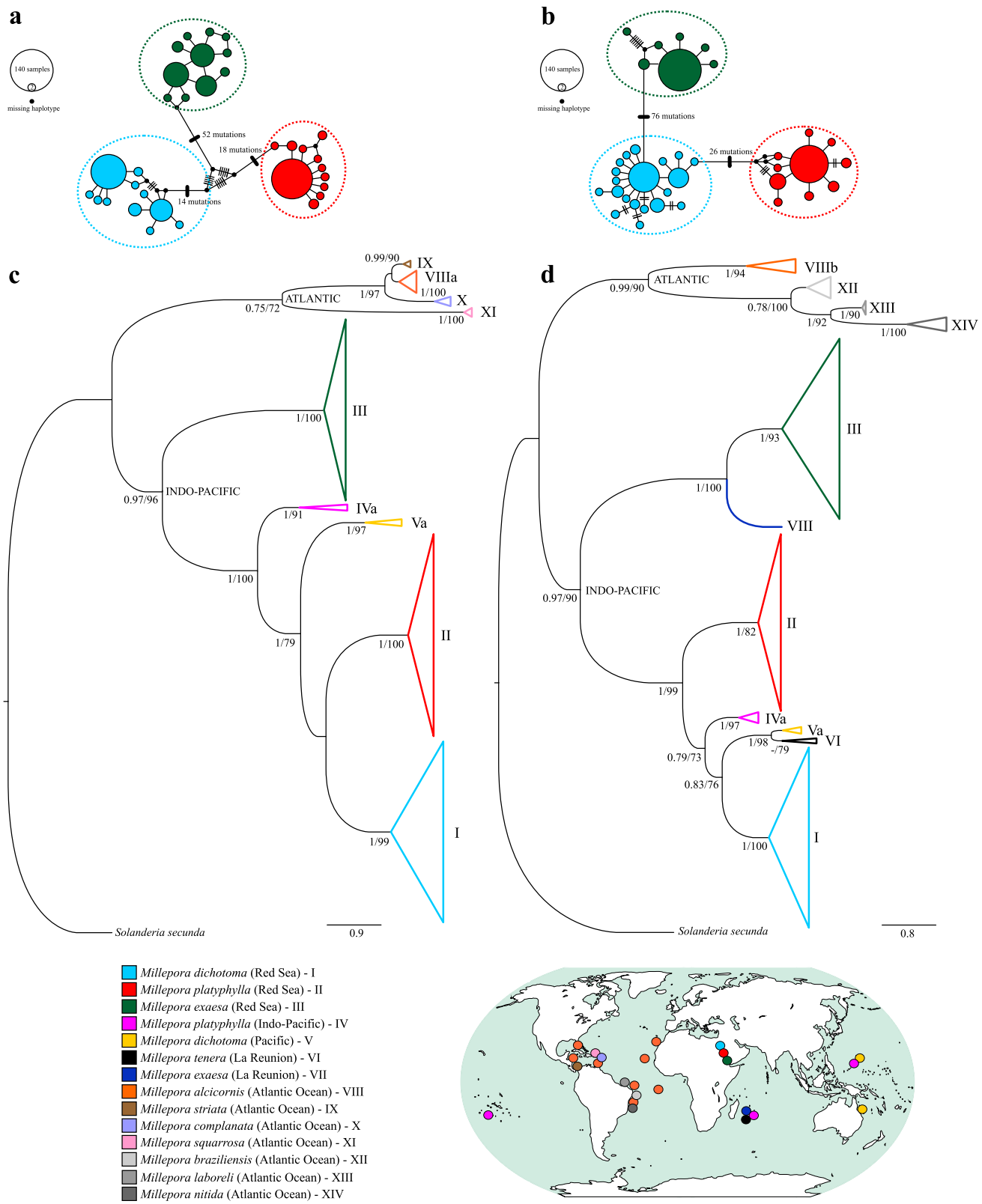


Fig. 3 **a** Pore characters analyzed in this study, gastropore diameter (1), dactylopore diameter (2), distance between gastropores (3), distance between dactylopores (4), distance from gastropore to nearest dactylopore (5), and number of dactylopores per gastropore (6). **b–c** Skeleton surface of the three *Millepora* species occurring in the Red Sea. **b** *M. dichotoma*; **c** *M. platyphylla*; **d** *M. exaesa*. Scale bars: 2 mm


a
140 samples
missing haplotype
52 mutations
18 mutations
14 mutations
b
140 samples
missing haplotype
76 mutations
26 mutations
c
0.99/90
IX
VIIIa
1/100
X
XI
1/100
ATLANTIC
1/97
0.75/72
III
1/100
INDO-PACIFIC
0.97/96
IVa
1/91
Va
1/97
II
1/100
1/79
I
1/99
Solanderia secunda
0.9
d
VIIIb
1/94
ATLANTIC
0.99/90
XII
0.78/100
XIII
1/90
1/92
XIV
1/100
III
1/93
1/100
VIII
INDO-PACIFIC
0.97/90
II
1/82
1/99
IVa
1/97
Va
VI
1/98
-/79
0.79/73
0.83/76
I
1/100
Solanderia secunda
0.8
Millepora dichotoma (Red Sea) - I
Millepora platyphylla (Red Sea) - II
Millepora exaesa (Red Sea) - III
Millepora platyphylla (Indo-Pacific) - IV
Millepora dichotoma (Pacific) - V
Millepora tenera (La Reunion) - VI
Millepora exaesa (La Reunion) - VII
Millepora alcicornis (Atlantic Ocean) - VIII
Millepora striata (Atlantic Ocean) - IX
Millepora complanata (Atlantic Ocean) - X
Millepora squarrosa (Atlantic Ocean) - XI
Millepora braziliensis (Atlantic Ocean) - XII
Millepora laboreli (Atlantic Ocean) - XIII
Millepora nitida (Atlantic Ocean) - XIV

of *M. dichotoma* from Northern Mariana Islands (de Souza et al. 2017), and clade VII grouping *M. exaesa* from Réunion Island. These molecular divergences between the Red Sea populations and the Southwestern Indo-Pacific ones of *M. dichotoma*, *M. platyphylla*, and *M. exaesa* were also clearly highlighted by the high genetic distances among these clades (Tables 1, 2). Indeed, the COI genetic distances between Red Sea *M. dichotoma* (clade I) and the one from Australia (clade Va) (9.17 ± 1.22%) and between Red Sea *M. platyphylla* (clade II) and the one from French Polynesia (clade IVa) (10.78 ± 1.34%) were comparable to the one between Red Sea *M. dichotoma* (clade I) and Red Sea *M. platyphylla* (clade II) (9.08 ± 1.32%). Similarly, the genetic distances based on 16S rDNA between Red Sea *M. dichotoma* (clade I) and the one from Northern Mariana Islands (clade Vb) (4.3 ± 0.81%), Red Sea *M. platyphylla* (clade II) and the one from Réunion Island and Northern Mariana Islands (clade IVb) (5.4 ± 0.92%), and Red Sea *M. exaesa* (clade III) and the one from Réunion Island (clade VII) (5.14 ± 1.01%) were comparable to the one between Red Sea *M. dichotoma* (clade I) and Red Sea *M. platyphylla* (clade II) (5.76 ± 0.97%).

Morphological results

The measurements of the eight pore characters are summarized in Table 3. Overall, *M. dichotoma* is characterized by small gastropore diameter (0.12 ± 0.02%), large distance between dactylopores (0.3 ± 0.06%), small number of dactylopores per gastropore (3.9 ± 0.89%), and small dactylopore density (24.39 ± 5.39%). *Millepora platyphylla* has small dactylopore diameter (0.04 ± 0.01%), large distance between gastropores (0.97 ± 0.17%), small distance from gastropore to nearest dactylopore (0.11 ± 0.02%), and an intermediate dactylopore density (43.71 ± 7.97%). *Millepora exaesa* shows a high number of dactylopores per gastropore (6.93 ± 0.84%), small gastropore density (5.27 ± 1.54%), and high dactylopore density (54.86 ± 10.7%).

The analysis of the three *Millepora* species revealed the presence of a common set of nematocysts shared by all species, with some species-specific variations. Regarding the polyp stage, all species showed the presence of small and large stenoteles and macrobasic apotrichous heteronemes (Fig. 5a–o). The heteronemes could be further classified as mastigophores and euryteles. Specifically, mastigophores were found in *M. dichotoma* (Fig. 5c–e) and *M. platyphylla* (Fig. 5h–j), whereas *M. exaesa* was characterized by euryteles (Fig. 5m–o). Regarding the eumedusoid stage, all fire coral species displayed the presence of large stenoteles in the bulbs but differences were found in the presence and the distribution of microbasic mastigophores on the exumbrella (Fig. 5p–w). *Millepora dichotoma* showed mastigophores grouped in a band in the middle portion of the exumbrella (Fig. 5p), *M. platyphylla* had mastigophores grouped in the lower part of the exumbrella, in between bulbs (Fig. 5s), and *M. exaesa* had an exumbrella deprived of nematocysts (Fig. 5v).

The MANOVA was run to test for differences in the multivariate pore characters measured for the three species of *Millepora* from the Red Sea. All but one pore characters were assessed. The differences among the three species based on the combined dependent variables were statistically significant ($F_{2,294} = 178{,}395$, $p < 0.0005$; Wilks' lambda 0.036, partial η^2 0.811). The follow-up univariate ANOVAs showed that all pore characters were statistically significantly different among the three analyzed species (Table 4). The Wilks' lambda test of discriminant function analysis showed significant differences in morphometric measurements for all the three species ($p < 0.001$) (Table S4). In the discriminant function analysis, the first DF (discriminant function) accounted for 66.6% and the second DF accounted for 33.4%, showing clear interspecific differentiation. The dactylopore density, number of dactylopores per gastropore, dactylopore distance, and gastropore diameter contributed to first DF while dactylopore diameter, distance between gastropores, and distance from gastropore to nearest dactylopore contributed to second DF (Table 5), indicating that the former four pore characters were the most important measurements in discriminating the three fire coral species. The DFA using cross-validation classification showed 99% correct classification of the analyzed individuals into their original species. Indeed, the proportion of correctly classified *M. dichotoma* samples was the highest (100%), followed by *M. exaesa* samples (99%) and *M. platyphylla* samples (98%). In accordance with the species identification, the DF I vs. DF II graphs depicted the presence of three units despite the presence of limited overlap between *M. exaesa* and *M. platyphylla* (Fig. 6). The analyses of the nematocysts measurements revealed statistically significant differences among the three fire coral species for the large stenoteles in both the polyp and the eumedusoid stages (Table 6), as well as for the small stenoteles and heteronemes in the polyp stage (Table 7).

Discussion

Our results confirmed the presence of three distinct species of *Millepora* in the Red Sea, i.e., *M. dichotoma*, *M. exaesa*, and *M. platyphylla*, based on new molecular and morphological data. The two sequenced mitochondrial genes (COI and 16S rDNA) clearly resolved the three species as distinct lineages and provided a robust phylogenetic

Table 3 List of pore and nematocyst characters analyzed in this study

Morphological feature	*M. dichotoma*	*M. platyphylla*	*M. exaesa*
Skeleton			
Gastropore diameter	0.12 ± 0.02 (0.09–0.17)	0.15 ± 0.02 (0.11–0.21)	0.14 ± 0.02 (0.1–0.2)
Dactylopore diameter	0.06 ± 0.01 (0.04–0.08)	0.04 ± 0.01 (0.02–0.07)	0.06 ± 0.01 (0.04–0.08)
Distance between gastropores	0.69 ± 0.17 (0.35–1.3)	0.97 ± 0.17 (0.68–1.46)	0.68 ± 0.12 (0.38–1.02)
Distance between dactylopores	0.3 ± 0.06 (0.19–0.48)	0.16 ± 0.04 (0.09–0.29)	0.15 ± 0.05 (0.05–0.32)
Distance from gastropore to nearest dactylopore	0.15 ± 0.04 (0.1–0.27)	0.11 ± 0.02 (0.06–0.16)	0.16 ± 0.03 (0.05–0.22)
Number of dactylopores per gastropore	3.9 ± 0.89 (1–6)	6.06 ± 0.96 (4–8)	6.93 ± 0.84 (5–9)
Gastropore density (0.25 mm^2)	5.71 ± 1.6 (3–11)	5.63 ± 2.22 (2–14)	5.27 ± 1.54 (2–9)
Dactylopore density (0.25 mm^2)	24.39 ± 5.39 (13–39)	43.71 ± 7.97 (24–61)	54.86 ± 10.7 (35–78)
Polyp			
Large stenotele length	16.9 1.53 (14.4–20.33)	16.3 ± 1.33 (14.24–19.11)	18.92 ± 2.84 (14.74–25.51)
Large stenotele width	13.3 ± 0.98 (11.54–14.88)	13.01 ± 1.04 (10.82–14.6)	15.6 ± 2.04 (12.49–20.21)
Small stenotele length	7.72 ± 0.67 (6.67–9.85)	9.16 ± 0.84 (7.93–11.02)	10.03 ± 0.75 (8.28–12.85)
Small stenotele width	6.02 ± 0.63 (5.19–8)	7.04 ± 0.95 (5.35–9.4)	7.69 ± 0.68 (5.65–9.43)
Heteroneme type	Mastigophore	Mastigophore	Eurytele
Heteroneme length	27.91 ± 1.4 (26.06–32.95)	28.88 ± 1.67 (26.78–34.67)	31.55 ± 2.06 (27.46–35.29)
Heteroneme width	22.66 ± 1.27 (20.28–25.96)	22.14 ± 1.32 (19.97–25.7)	22.91 ± 1.67 (18.39–25.97)
Heteroneme shaft	162.33 ± 15.72 (140.38–190.25)	410.8 ± 15.91 (394.17–445.15)	331.34 ± 19.58 (307.21–367.15)
Eumedusoid			
Stenotele length	14.42 ± 0.71 (13.03–16.08)	14.37 ± 0.9 (12.76–16.47)	14.78 ± 0.92 (13.14–17.35)
Stenotele width	11.54 ± 0.73 (9.48–12.76)	11.99 ± 0.66 (10.47–13.33)	12.42 ± 0.81 (9.98–14.67)
Mastigophore length	11.82 ± 0.83 (10.16–14.13)	10.83 ± 0.51 (9.3–11.9)	Absent
Mastigophore width	9.56 ± 0.73 (7.6–11.21)	9.06 ± 0.33 (8.27–9.87)	Absent
Mastigophore area	88.89 ± 10.56 (64.53–107.2)	77.16 ± 5.22 (60.4–89.15)	Absent
Mastigophore ratio	1.24 ± 0.1 (1.04–1.49)	1.2 ± 0.06 (1.05–1.4)	Absent
Medusa size length	740.45 ± 68.32 (645.4–849.58)	765.01 ± 40.15 (717.38–822.85)	648.58 ± 47.44 (592.03–730.13)
Medusa size width	765.72 ± 91.18 (679.86–906.01)	749.65 ± 32.99 (718.31–791.22)	647.59 ± 40.72 (583–703.14)
Medusa size ratio	0.97 ± 0.05 (0.91–1.04)	1.02 ± 0.03 (0.99–1.06)	1.01 ± 0.11 (0.87–1.18)

Mean values, standard deviations, and ranges of these features obtained from the three Red Sea *Millepora* species

hypothesis of the genus. Based on pore characters, the analyzed samples were grouped in three clusters in full agreement with the in situ species identification. Finally, significant differences in the nematocysts of both polyps and eumedusoids examined for the first time in material from this region were found among the three analyzed species of fire corals from the Red Sea.

Although early taxonomic works suggested the presence of a single widespread species of *Millepora* throughout the Indo-Pacific and the Caribbean-Atlantic (Hickson 1898, 1809), several taxonomists recognized three well-defined species of fire corals in the Red Sea, i.e., *M. dichotoma*, *M. exaesa*, and *M. platyphylla* (Klunzinger 1879; Crossland 1941; Boschma 1948a, b, 1966). These authors stated that these species were clearly distinct based on the colony growth form and provided a detailed description of their morphological characters. Our study confirmed these findings and included innovative information about the genetics, the skeletal pores, and the nematocysts of the three species, discussed hereafter.

The resolution of the species boundaries in the Red Sea fire corals using a combination of genetic data and morphology at two distinct levels, i.e., skeletal pore and nematocysts, showed that an integrated morpho-molecular approach is useful for a better understanding of the systematics of *Millepora*. Our phylogeny reconstructions based on COI and 16S rDNA resolved well-supported clades within this genus of hydrozoans, and for the first

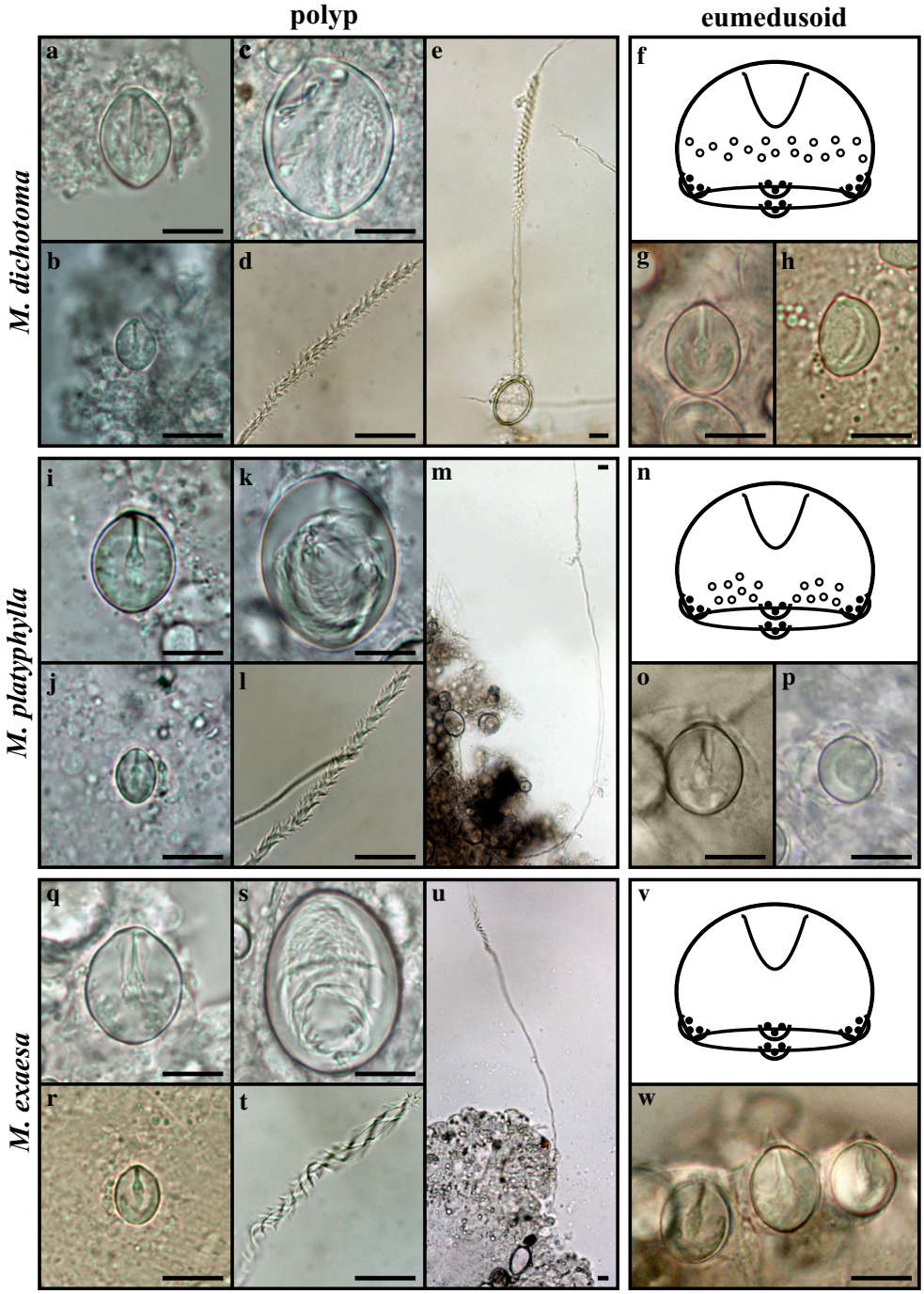


Fig. 5 Cnidome of the polyp (**a**–**o**) and eumedusoid stages (**p**–**w**) of the three *Millepora* species analyzed in this work. **a** Large and **b** small undischarged stenoteles, **c** macrobasic apotrichous mastigophores undischarged capsule, **d** detail of the discharged distal portion of the shaft, and **e** discharged capsule in *M. dichotoma*. **f** Large and **g** small stenoteles, **h** macrobasic apotrichous mastigophores undischarged capsule, **i** detail of the discharged distal portion of the shaft, and **j** discharged capsule in *M. platyphylla*. **k** Large and **l** small stenoteles, **m** macrobasic apotrichous euryteles undischarged capsule, **n** detail of the discharged distal portion of the shaft, and **j** discharged capsule in *M. exaesa*. **p** Nematocyst distribution, **q** stenoteles, and **r** microbasic mastigophores in the eumedusoid of *M. dichotoma*. **s** Nematocyst distribution, **t** stenoteles, and **u** microbasic mastigophores in the eumedusoid of *M. platyphylla*. **v** Nematocyst distribution, and **w** stenoteles in the eumedusoid of *M. exaesa*. Black and white dots in Figs. p, s, v represent stenoteles and microbasic mastigophores, respectively. Scale bars: 10 μm

time, these two molecular markers were used to build a phylogenetic hypothesis of the Indo-Pacific fire corals. In fact, the former locus successfully resolved the species boundaries among *M. alcicornis*, *M. complanata*, *M.*

Table 4 One-way ANOVA results for differences in the analyzed pore characters among the three species of *Millepora* from the Red Sea

Pore character	Sum of squares	*df*	Mean square	*F*	Sign.	Partial eta η^2
Gastropore diameter	0.063	2	0.031	77.011	0.000	0.341
Dactylopore diameter	0.029	2	0.014	206.020	0.000	0.581
Distance between gastropores	5.463	2	2.731	112.733	0.000	0.432
Distance between dactylopores	0.150	2	0.075	240.462	0.000	0.618
Distance from gastropore to nearest dactylopore	0.018	2	0.009	71.856	0.000	0.326
Number of dactylopores per gastropore	486.780	2	243.390	299.759	0.000	0.669
Dactylopore density	47,533.527	2	23,766.763	343.049	0.000	0.698

Table 5 Contribution of each pore character to the discriminant functions for the three species of *Millepora* from the Red Sea

Pore character	DF1	DF2
Dactylopore density	0.579*	0.317
Number of dactylopores per gastropore	0.558*	0.229
Distance between dactylopores	− 0.520*	− 0.014
Distance between gastropores	0.281*	− 0.122
Dactylopore diameter	− 0.229	0.597*
Gastropore diameter	0.175	− .438*
Distance from gastropore to nearest dactylopore,	− 0.118	0.365*

*Largest correlation between each variable and any DF

squarrosa, and *M. striata* from the Caribbean (Ruiz-Ramos et al. 2014). The latter has been already used by de Souza et al. (2017) to define the phylogenetic relationships and the connectivity of the Caribbean-Atlantic fire corals. The resulting phylogenetic hypothesis successfully resolved all the analyzed species, *i.e.*, *M. alcicornis*, *M. braziliensis*, *M. laboreli*, and *M. nitida* (de Souza et al. 2017). Therefore, COI and 16S rDNA may be selected for future works aimed to evaluate the species boundaries of *Millepora* throughout its entire geographic distribution. A potential additional marker could be the ITS region that has been successfully used for the identification of *Millepora* from Japan (Takama et al. 2018) The recently developed set of 15 microsatellites Dubé et al. (2017c) seemed, however, to be only valid for the Indo-Pacific species.

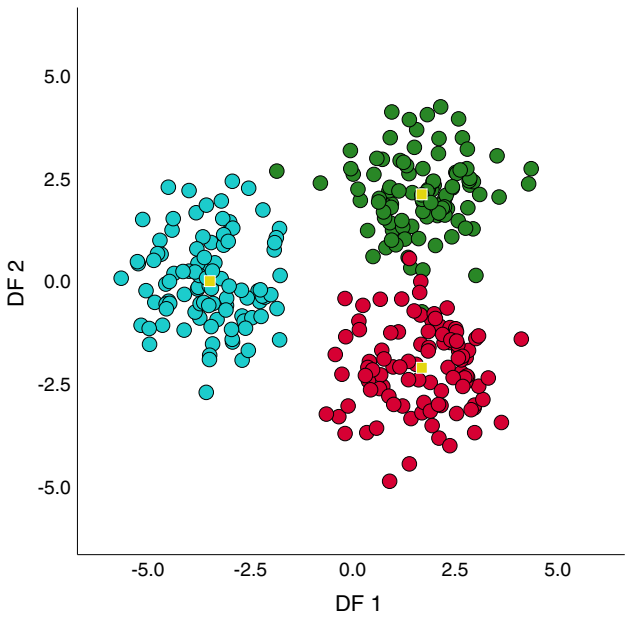


Fig. 6 Discriminant analysis plot for the analyzed pore characters of *Millepora* species from Red Sea. *Millepora dichotoma* (blue), *M. platyphylla* (red), and *M. exaesa* (green)

Pores have been usually considered extremely variable, and their use as informative taxonomic characters has been criticized by several taxonomists (Hickson 1898, 1809; Crossland 1948; Boschma 1948a, 1966). Nevertheless, Moshchenko (1994, 1995b, 1996b) examined in a rigorous way the utility of several pore characters, including the ones we analyzed in the present work, and concluded that pores were able to define *Millepora* species. Indeed, a quantitative evaluation of pore characters in fire corals from Vietnam successfully discriminated *M. platyphylla* from the five branching species occurring in that area, i.e., *M. cruzi* Nemenzo, 1975 (a synonym of *M. intricata*; see Razak and Hoeksema 2003), *M. dichotoma*, *M. intricata*, *M. murrayi* Quelch, 1884, and *M. tenera*, while a continuous gradient was found among the branching species (Moshchenko 1994, 1995b, 1996b). These results were further confirmed by investigations of growth forms, enzymes, and anatomy of the skeleton (Moshchenko

Table 6 One-way ANOVA results for differences in the polyp and eumedusoid large stenoteles among the three species of *Millepora* from the Red Sea

Character	Medusa large stenotele					Polyp large stenotele				
	Sum of squares	*df*	Mean squares	*F*	Significance	Sum of squares	*df*	Mean squares	*F*	Significance
Length	4.851	2	2.426	3.238	0.042	119.627	2	59.813	14.444	0.000
Width	16.492	2	8.246	14.975	0.000	127.295	2	63.647	30.127	0.000
Area	4282.191	2	2141.095	9.258	0.000	86,947.724	2	43,473.862	22.059	0.000
Ratio	0.083	2	0.042	9.985	0.000	0.059	2	0.030	6.031	0.003

Table 7 Kruskal–Wallis test results for differences in the polyp small stenoteles and heteronemes among the three species of *Millepora* from the Red Sea

Character	Small stenotele			Heteroneme		
	H Kruskal–Wallis	*df*	Significance	H Kruskal–Wallis	*df*	Significance
Length	99.577	2	0.000	69.526	2	0.000
Width	71.654	2	0.000	12.026	2	0.002
Area	88.231	2	0.000	31.020	2	0.000
Ratio	2.319	2	0.314	87.219	2	0.000

1994, 1995b, 1996b). Interestingly, a recent molecular study demonstrated that the three branching species of fire corals found in Japan, i.e., *M. dichotoma*, *M. intricata*, and *M. tenera*, were nested together in a single molecular clade (Takama et al. 2018). More generally, Takama et al. (2018) recovered four molecular lineages of *Millepora* that were not in agreement with the traditional morphology-based species identification (Razak and Hoeksema 2003). Nevertheless, they also showed significant differences in gastropore diameter, dactylopore diameter, distances between gastropores among the genetic clades, with the only exception of the absence of significant differences between two clades. Overall, based on our results and the published studies, we conclude that pores are informative to tell apart at least some of the molecular clades of *Millepora*.

The cnidome of *Millepora*, i.e., type, size, and distribution of nematocysts, was scarcely investigated in the past, with a few exceptions. For instance, a first detailed assessment of the polyp nematocysts was presented by Calder (1988), whose description of *Millepora alcicornis* from Bermuda included information and images of stenotele and mastigophore capsules. More recently, other authors partially investigated the cnidome of a few *Millepora* species from both Atlantic and Indo-Pacific localities (Meroz-Fine et al. 2003; García-Arredondo et al. 2012; Bourmaud et al. 2013), finding in some cases differences in the size and the distribution of the nematocysts among fire coral morphotypes (Meroz-Fine et al. 2003; Bourmaud et al. 2013). In this work, we analyzed and compared for the first time the complete set of the nematocysts found in both the polyp and the eumedusoids stages of three *Millepora* species from the Red Sea, finding significant differences among them and demonstrating the utility of the cnidome in species discrimination within *Millepora* hydrozoans. Not only the size, but also the distribution of the nematocysts of Red Sea *Millepora* species differed among taxa. Indeed, similarly to the observations of Bourmaud et al. (2013) on eumedusoids from Réunion Island, we found that the eumedusoids of the three Red Sea species showed a different organization of the mastigophores on the exumbrella. *Millepora exaesa* eumedusoids from both the Red Sea and Réunion Island lacked mastigophores, and this morphological similarity is also supported by the fact that these two taxa clustered together in a fully supported clade in the 16S rRNA phylogeny reconstruction. Moreover, *M. dichotoma* from the Red Sea and *M. tenera* from Réunion Island showed a similar pattern of exumbrellar nematocyst organization and indeed are closely related in the 16S rRNA phyogenetic tree. Contrarily, *M. platyphylla* from the Red Sea and Réunion Island had mastigophores organized in different ways and was phylogenetically divergent.

Our single-gene phylogeny reconstructions revealed unexpected phylogenetic patterns for fire corals in the Indo-Pacific. Although the genetic analyses of our samples confirmed the presence of three lineages at the species level within the Red Sea, the inclusion of available *Millepora* sequences from the Indo-Pacific in our analyses resulted in the polyphyly of both *M. dichotoma* and *M. platyphylla* and in the presence of two divergent and allopatric lineages of *M. exaesa*. Unfortunately, no specimen photographs were published for these sequences downloaded from GenBank

with the only exception of the samples of *M. dichotoma* from Australia included in the COI phylogeny reconstruction (Schweinsberg et al. 2016). Nevertheless, *M. platyphylla* and *M. exaesa* are both supposedly morphologically distinctive species in the Indo-Pacific as they are told apart based on their unique colony growth form (Boschma 1949; Razak and Hoeksema 2003), although a certain degree of ecophenotypic variation occurs, especially in *M. platyphylla* (Dubé et al. 2017a, b). Therefore, we are prone to reject the hypothesis of a misidentification by the authors based on colony morphology of both *M. platyphylla* from Réunion Island and French Polynesia (Leray et al. 2013; Schweinsberg et al. 2016) and *M. exaesa* from Réunion Island. This being said, the deep intraspecific genetic divergences between populations of *M. dichotoma*, *M. exaesa*, and *M. platyphylla* from the Red Sea and various localities of the Southwestern Indo-Pacific suggested that these populations represented distinct evolutionary units that have not been detected in previous taxonomic works. These three species have been historically considered widespread throughout the Indo-Pacific, from the Red Sea to French Polynesia (Klunzinger 1879; Crossland 1941, 1948; Boschma 1949; Razak and Hoeksema 2003), but our genetic analyses clearly demonstrated that they all constitute species complexes. Each of these three species complexes is composed of at least two species that share a similar colony growth form. Therefore, while the growth of the colony is a diagnostic character to distinguish the Red Sea species between each other (Forskål 1775; Ehrenberg 1834; Klunzinger 1879), it is clearly not informative to define boundaries within the three species complexes. The genetic distances between the two distinct lineages for each of the three nominal species are comparable to those occurring between valid species in the genus for the examined markers (Tables 1, 2). Given that the type locality of *M. dichotoma*, *M. exaesa*, and *M. platyphylla* is the Red Sea, a future taxonomic revision of *Millepora* would maintain these names for the three Red Sea molecular lineages while new names or synonymized names would be assigned to the other Indo-Pacific lineages of these three species. In this respect, it will be relevant to re-examine *Millepora tuberosa* Boschma, 1966, since its type specimens from Mauritius resemble corals of *M. exaesa*. Boschma (1966) considered *M. exaesa* a doubtful species, and he suspected that specimens from outside the Red Sea are actually *M. tuberosa.* Both taxa were synonymized by Razak and Hoeksema (2003), who gave priority to *M. exaesa*, being the senior synonym. Since Réunion Island is only 225 km away from Mauritius, this may also concern specimens from this locality that were studied by Dubé et al. (2017c), who did not refer to Boschma's (1966) work. Our results remove the doubts expressed by Boschma (1966) concerning the status of *M. exaesa* as a valid species.

From a morphological point of view, either morphological convergence of the colony growth form or character state conservation from the ancestor may have occurred. On the base of our phylogenetic trees, the former hypothesis may be applied to *M. dichotoma* and *M. platyphylla*, given the absence of a sister relationships between the Red Sea clades and the Southwestern Indo-Pacific ones. The second explanation is more likely for *M. exaesa* considering that the two clades from the Red Sea and Réunion Island are sister taxa, possibly *M. exaesa* and *M. tuberosa*. Despite the absence of variation in the colony growth form, our integrated morphological analyses on Red Sea *Millepora* suggests the need to further evaluate both pore structures and nematocysts of the Southwestern Indo-Pacific clades to evaluate the occurrence of possible differences of these morphological traits between the Red Sea and the Southwestern Indo-Pacific molecular lineages. Indeed, nematocysts of *M. dichotoma*, *M. exaesa*, and *M. platyphylla* from the Southwestern Indo-Pacific have never been studied. Moreover, no comparisons of pores between the Red Sea and the Southwestern Indo-Pacific colonies of these three species have been undertaken so far. From a biological point of view, the distinction between the Red Sea and the Southwestern Indo-Pacific clades may suggest that either the Red Sea lineages have a small-distance dispersal capability or the Southwestern Indo-Pacific lineages have failed to disperse within the Red Sea. At least for the case of clade IVb (*M. platyphylla* from Réunion Island and Northern Mariana Islands), this evolutionary unit is capable of long-distance dispersal. This contrasting pattern of short- and long-distance dispersal capabilities among species is similar to the one occurring in the Caribbean-Atlantic where *M. alcicornis* showed a high connectivity within the Caribbean while the three endemic species of Brazil, i.e., *M. braziliensis*, *M. nitida*, and *M. laboreli*, are restricted to few locations and isolated by strong biogeographic barriers (de Souza et al. 2017).

A growing number of studies have revealed that several hydrozoans traditionally considered to display a large geographic distribution are indeed species complexes composed of geographically circumscribed lineages (*e.g.*, Schuchert 2014; Postaire et al. 2016, 2017; Maggioni et al. 2017b; Montano et al. 2017). For example, the near-cosmopolitan species *Plumularia setacea* (Linnaeus, 1758) is subdivided in a multitude of distinct molecular clades showing distinct geographic distributions, such as South Africa, the Western Indian Ocean, the Caribbean, and the Mediterranean Sea. Similarly, Postaire et al. (2017) revealed a deep and strong genetic differentiation between the Indian and the Pacific populations of *Macrorhynchia phoenicea* (Busk 1852). Several analogous cases have been

recently described in many hard corals throughout the Indo-Pacific (Flot et al. 2008; Ladner and Palumbi 2012; Pinzón et al. 2013; Richards et al. 2016; Gélin et al. 2017; Arrigoni et al. 2016a). Moreover, the existence of unexpected intraspecific evolutionary breaks between the Red Sea and the Southwestern Indo-Pacific populations/species has been discovered based on genetic studies in several marine phyla, including scleractinian corals (Arrigoni et al. 2012, 2016b; Huang et al. 2014), octocorals (Reijnen et al. 2014), sea urchins (Bronstein et al. 2016), and fishes (DiBattista et al. 2013; Fernandez-Silva et al. 2015; Ahti et al. 2016; Waldrop et al. 2016). In most cases, these genetic divergences between oceans reflect the importance of geological features, such as tectonic plates and mantle plume tracks. Indeed, it is now largely accepted that the actual distribution of most hard corals depends more on geological events than contemporary environmental conditions and habitats (Keith et al. 2013; Leprieur et al. 2016). For example, important tectonic changes in what is now the Indian Ocean occurred during both the Eocene and Oligocene, resulting in the fragmentation of the Tethys Sea (Schettino and Turco 2011) and may have promoted isolation and speciation evens.

In conclusion, the obtained results clearly resolved the species boundaries of *Millepora* in the Red Sea. Given the long-term challenges of morphology-based species identification of these organisms (Hickson 1898; Boschma 1948b, 1949) and the recent unexpected molecular findings in discordance with traditional systematics (Ruiz-Ramos et al. 2014; Takama et al. 2018), we believed that the presented approach may be useful to define the relationships among the fire corals. Moreover, the recovery of multiple molecular lineages of *M. dichotoma*, *M. exaesa,* and *M. platyphylla* between the Red Sea and the Indo-Pacific suggested the need to investigate more material from additional localities of the Indo-Pacific. The integration of genetics and morphology (pore structure and nematocysts of both polyp and eumedusoid stages) may be the key to elucidate what Boschma (1948a) defined "the species problem in *Millepora*" more than 70 years ago.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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