RESEARCH ARTICLE

The Hidden Diversity of Zanclea Associated with Scleractinians Revealed by Molecular Data

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Abstract

Scleractinian reef corals have recently been acknowledged as the most numerous host group found in association with hydroids belonging to the Zanclea genus. However, knowledge of the molecular phylogenetic relationships among Zanclea species associated with scleractinians is just beginning. This study, using the nuclear 28S rDNA region and the fast-evolving mitochondrial 16S rRNA and COI genes, provides the most comprehensive phylogenetic reconstruction of the genus Zanclea with a particular focus on the genetic diversity among Zanclea specimens associated with 13 scleractinian genera. The monophyly of Zanclea associated with scleractinians was strongly supported in all nuclear and mitochondrial phylogenetic reconstructions. Furthermore, a combined mitochondrial 16S and COI phylogenetic tree revealed a multitude of hidden molecular lineages within this group (Clades I, II, III, V, VI, VII, and VIII), suggesting the existence of both host-generalist and genus-specific lineages of Zanclea associated with scleractinians. In addition to Z. gallii living in association with the genus Acropora, we discovered four well-supported lineages (Clades I, II, III, and VII), each one forming a strict association with a single scleractinian genus, including sequences of Zanclea associated with Montipora from two geographically separated areas (Maldives and Taiwan). Two host-generalist Zanclea lineages were also observed, and one of them was formed by Zanclea specimens symbiotic with seven scleractinian genera (Clade VIII). We also found that the COI gene allows the recognition of separated hidden lineages in agreement with the commonly recommended mitochondrial 16S as a DNA barcoding gene for Hydrozoa and shows reasonable potential for phylogenetic and evolutionary analyses in the genus Zanclea. Finally, as no DNA sequences are available for the majority of the nominal Zanclea species known, we note that they will be necessary to elucidate the diversity of the Zanclea-scleractinian association.

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Introduction

Hydroids belonging to the genus *Zanclea* Gegenbaur, 1857 (Cnidaria, Hydrozoa) are distributed worldwide [1–5] and can be found from the intertidal zone [6–8] up to a depth of 500 m [9]. Of all 34 nominal species ascribed to this genus, a dozen have been described exclusively based on medusa specimens collected using plankton nets [10–14]. The remaining *Zanclea* species, identified through observation of both polyp and medusa stages, are known to have a preference for living substrates, usually forming symbiotic relationships with marine organisms such as bivalves, octocorals and bryozoans [5, 15–20]. Scleractinian reef corals are traditionally known to host many taxa of associated organisms [21, 22]; recently, several studies have revealed that the genus *Zanclea* is an additional component of this plethora of symbioses [6–8, 15].

After a few restricted preliminary reports from Mozambique [23, 24] and Papua New Guinea [15], an increasing number of studies on *Zanclea*-scleractinian symbiosis have recently been published focusing on different aspects of this close association such as ecology, taxonomy, physical interactions, and geographical distribution [6–8, 25–29]. The association with scleractinians currently involves the four species *Zanclea gilii* Boero, Bouillon & Gravili, 2000; *Zanclea margaritae* Pantos & Bythell, 2010; *Zanclea sango* Hirose & Hirose, 2011; and *Zanclea gallii* Montano, Maggioni & Puce 2014 and some as yet unidentified species [6–8, 15, 26–30]. All those species belong to the “polymorpha group” showing colonies of hydroids consisting of both retractile gastro-gonozooids and dactylozooids [15]. The geographic distribution of this association includes the Red Sea [27] and several Indo-Pacific regions such as Australia, Indonesia, Taiwan, Japan and the Republic of Maldives [7, 8, 26, 30]. The host range currently includes approximately 24 scleractinian genera belonging to 7 families, with a total of 33 scleractinian species involved [29]. Thus, reef-building corals are the host group with the highest number of species found in association with *Zanclea* species.

Fontana et al. [26] recently proposed a genus-specific association between *Zanclea* and scleractinians. However, whereas *Z. gilii*, *Z. margaritae*, and the unidentified *Zanclea* specimens studied by Fontana et al. [26] settle locally on genus *Acropora* [6, 28], *Z. sango* is a more generalist species living on the genera *Pavona* and *Psammocora* and it shows a widespread distribution [28]. Unfortunately, except for these preliminary data, no other information at the species level is available regarding the host-specificity and diversity of *Zanclea* associated with scleractinians. Differences in the hydroid colony, the absence and presence of perisarc and the cnidome of both the polyp and medusa stages are the morphological features generally used to identify *Zanclea* species [7, 15, 16, 19, 28, 31]. Considering that the diversity of this genus, as well as of many cnidarians, could be underestimated due to the difficulty of morphologic identification, molecular techniques, as part of an ‘integrated taxonomy’ approach [32], may be very useful.

Knowledge regarding the molecular phylogenetic relationships among *Zanclea* species associated with scleractinians is still far from complete. In fact, with the exception of the recent description of *Z. gilii* based on an integrated morpho-molecular approach [28], the other three *Zanclea* species have been described only through the study of their morphological characters [6, 7, 15]. At present, mitochondrial and nuclear phylogenetic analyses have shown that all the available sequences of *Zanclea* associated with scleractinians form a monophyletic lineage clearly separated from the genus type species *Zanclea costata* Gegenbaur, 1857 [26, 28]. Within this cohesive group, both *Z. sango* and *Z. gilii* were recovered as distinct monophyletic lineages based on partial 16S gene sequences, with the latter species closely related but molecularly separated from the unidentified *Acropora*-associated *Zanclea* specimens studied by Fontana et al. [26, 28]. However, no sequences are currently available for *Z. gilii* and *Z. margaritae*.
The mitochondrial cytochrome c oxidase I (COI) gene has been broadly adopted as a barcoding gene for animal life [33, 34]. Nevertheless, its utility has been strongly criticized in some animals at the base of the Metazoan tree, such as Porifera and Cnidaria, due to the slow nucleotide substitution rate of the mitochondrial genome resulting in an overlap between intra- and interspecific divergence [35–37]. Concerning Hydrozoa, although in some cases this gene has been revealed as phylogenetically informative [38–40], the mitochondrial 16S rRNA gene has been preferentially used being highly variable, easy to amplify and useful for distinguishing nominal and cryptic hydroid species [28, 41–46]. For these reasons, the mitochondrial 16S gene has been proposed as a barcode across Hydrozoa [45].

Herein, we collected 63 specimens of *Zanclea* living on 13 scleractinian genera in Faafu Atoll, Maldives, which represents an area hosting a relatively high number of reef coral genera currently known to be involved in this symbiosis [29]. The genetic diversity and the phylogenetic relationships of *Z. sango*, *Z. gallii*, and several other unidentified *Zanclea* specimens associated with different scleractinian hosts were investigated by sequencing three molecular markers, the nuclear 28S rDNA region and the fast-evolving mitochondrial genes, 16S rRNA and COI genes, to evaluate the molecular diversity and degree of host specificity of *Zanclea* associated with scleractinians. Furthermore, we evaluated whether the COI gene is phylogenetically informative and appropriate among *Zanclea* species associated with scleractinians.

**Material and Methods**

**Sample collection**

The sampling was conducted between March and May 2014 in the waters around Magoodhoo Island, Faafu Atoll, Republic of Maldives (3°04’ N; 72°57’ E) (S1 Fig). The presence of *Zanclea* on scleractinian genera was recorded qualitatively *in situ*. Up to 13 scleractinian genera hosting *Zanclea* were selected and small fragments were collected for each of them. Single hydroid polyps were carefully collected one by one using a syringe needle directly from a bowl filled with seawater placed under a stereomicroscope. Afterwards, they were immediately preserved in 95% ethanol for further molecular analyses and fixed in 4% formalin for taxonomic identification. For documentary purposes we took underwater photographs of *Zanclea*-coral associations using a Canon G11 camera in a Canon WP-DC 34 underwater housing (Fig 1).

Microphotographs (32x) of hydroids protruding from the coral skeletons were taken by use of a Leica EZ4 D stereomicroscope equipped with a Canon G11 camera (Fig 1). All hydroids (except *Z. gallii* and *Z. sango*) were identified at genus level according to Bouillon et al. [47], while the scleractinian hosts were identified to genus level according to updated taxonomic classifications: Acroporidae [48, 49], Agariciidae [50], Dendrophylliidae [51, 52], Lobophylliidae [53, 54], Merulinidae [53, 55, 56], Poritidae [57].

**Ethics Statement**

The field study was approved by the Ministry of Fisheries and Agriculture of the Republic of Maldives and it did not involve endangered or protected species.

**Molecular analyses**

The total genomic DNA of 63 ethanol-fixed *Zanclea* samples from 13 scleractinian genera was extracted following a protocol modified from Zietara et al. [58]. Three different molecular markers were amplified: (1) a ~300 bp portion of the nuclear 28S ribosomal DNA gene (28S), (2) a ~400 bp portion of the mitochondrial 16S ribosomal RNA gene (16S), and (3) a ~700 bp portion of the mitochondrial cytochrome oxidase subunit I gene (COI). The first two regions
of DNA have been extensively used to infer phylogenetic relationships among hydroids in numerous previous molecular studies [26, 28, 44, 45, 59–61]. We also selected the barcoding region of COI gene because it turned out to be useful for species delimitation in Hydrozoa [40, 62]. 16S and 28S genes were amplified using hydroid-specific primers and the protocols proposed by Fontana et al. [26]. The barcoding region of COI gene was amplified using universal primers LCO1490 and HCO2198 and the protocol proposed by Folmer et al. [63]. All PCR products were purified and directly sequenced in forward and reverse directions using

Fig 1. In situ photographs and microphotographs of living Zanclea hydroids associated with scleractinians. A) Goniiestrea; B) Porites; C) Montipora; D) Acropora; E) Pavona; F) Favites; G) Dipsastrea; H) Echinopora; I) Platygyra. (Scale bars: ~ 500 μm)
an ABI 3730xl DNA Analyzer (Applied Biosystem, Foster City, CA, USA). The sequences obtained in this study were deposited with the EMBL, and the accession numbers are listed in Table 1.

**Molecular phylogenetic analyses and haplotype network**

The chromatograms were viewed, edited, and assembled using CodonCode Aligner 3.7.0 (CodonCode Corporation, Dedham, MA, USA). Alignments of the three separate datasets were generated using the E-INS-i option in MAFFT 7.110 [64, 65] with default parameters. Genetic distances (Kimura 2-parameter) within and among nominal Zanclea species and/or our Zanclea molecular lineages were computed for each separated molecular locus using MEGA 6 [66].

To examine whether the sequences from 16S and COI loci should be combined in a single analysis, a partition-homogeneity test was run in PAUP 4.0b1 [67], and significance was estimated by 1000 repartitions. This test, described as the incongruence-length divergence test by Farris et al. [68], indicated no conflicting phylogenetic signals between the datasets (P = 0.99). Therefore, 16S and COI were linked and datasets from both molecular markers were concatenated into a single data matrix, while the 28S sequences were considered as a separate set. Single 16S and COI trees are reported in S2 and S3 Figs, respectively. The newly obtained 28S sequences of Zanclea were aligned with other homologous ones available in GenBank and DRYAD databases (DOI: http://dx.doi.org/10.5061/dryad.g0b20) and belonging both to the genus Zanclea and to other families of the clade Capitata [26, 28, 59, 60, 69] (Table 1). Hydra vulgaris, a representative of the clade Aplanulata [59], was selected as outgroup due to its divergence from the clade Capitata [60, 70]. For the concatenated 16S and COI dataset, our newly obtained Zanclea sequences were aligned with homologous sequences of Zanclea sp. available in GenBank and coming from China Sea and unknown host (Table 1). We selected these sequences because of their sister relationship with our scleractinian-associated Zanclea sequences as shown in the 28S analyses. Phylogenetic analyses were performed using three methods: Maximum Parsimony (MP), Bayesian Inference (BI), and Maximum Likelihood (ML). MP analyses were performed using PAUP4.0b10 with heuristic searches stepwise addition and tree-bisection-reconnection (TBR) branch swapping. The node consistency was assessed using 500 bootstrap replicates with randomly added taxa. The software MrModeltest2.3 [71] was used, in conjunction with PAUP4.0b10, to select the best-fit nucleotide substitution models for each locus. The most suitable models estimated using the Akaike information criterion (AIC) were GTR + I + for 28S, HKI + I + for 16S, and GTR + I for COI. BI analyses were performed using MrBayes 3.1.2 [72]. Four parallel Markov Chain Monte Carlo runs (MCMC) were conducted for 5 x 10^7 generations for 28S and COI loci, 6x 10^7 generations for 16S locus, and 6 x 10^7 for the combined 16S and COI loci. Trees were sampled every 100 generations for each analysis, and the initial 25% of the total trees were discarded as burn-in based on checking the parameter estimates and convergence using Tracer 1.5 [73]. ML trees were built with PhyML 3.0 [74] using the evolutionary models selected by MrModeltest2.3 and the robustness of each clade was tested using 500 bootstrap replications.

Finally, sequences were converted into the Roehl format using DnaSP 5 [75] and haplotype networks for separate 16S and COI datasets were constructed in Network 4.6.1.2 (http://www.fluxus-technology.com) using the median-joining algorithm [76] and default settings.
| Species    | Coral host genus | Specimen code | Locality | Genbank accession numbers |
|------------|------------------|---------------|----------|--------------------------|
| Zanclea gallii | Acropora      | MA056         | Maldives | LN714228 LN714105 LN714169 |
| Zanclea gallii | Acropora      | MA057         | Maldives | LN714229 LN714106 LN714170 |
| Zanclea gallii | Acropora      | MA058         | Maldives | LN714230 LN714107 LN714171 |
| Zanclea gallii | Acropora      | MA059         | Maldives | LN714231 LN714108 LN714172 |
| Zanclea gallii | Acropora      | AC1           | Maldives | LN794213 LK934472 LK934479 |
| Zanclea sango  | Pavona        | MA051         | Maldives | LN714225 LN714100 LN714164 |
| Zanclea sango  | Pavona        | MA052         | Maldives | LN714226 LN714101 LN714165 |
| Zanclea sango  | Pavona        | MA053         | Maldives | LN714227 LN714102 LN714166 |
| Zanclea sango  | Pavona        | MA054         | Maldives | LN714228 LN714103 LN714167 |
| Zanclea sp.    | Goniastrea    | MA001         | Maldives | LN714179 LN714050 LN714115 |
| Zanclea sp.    | Goniastrea    | MA002         | Maldives | LN714180 LN714051 LN714116 |
| Zanclea sp.    | Goniastrea    | MA003         | Maldives | LN714181 LN714052 LN714117 |
| Zanclea sp.    | Goniastrea    | MA004         | Maldives | LN714182 LN714053 LN714118 |
| Zanclea sp.    | Goniastrea    | MA005         | Maldives | LN714183 LN714054 LN714119 |
| Zanclea sp.    | Favites       | MA006         | Maldives | LN714184 LN714055 LN714120 |
| Zanclea sp.    | Favites       | MA007         | Maldives | LN714185 LN714056 LN714121 |
| Zanclea sp.    | Favites       | MA008         | Maldives | LN714186 LN714057 LN714122 |
| Zanclea sp.    | Favites       | MA009         | Maldives | LN714187 LN714058 LN714123 |
| Zanclea sp.    | Favites       | MA010         | Maldives | LN714188 LN714059 LN714124 |
| Zanclea sp.    | Dipsastrea    | MA011         | Maldives | LN714189 LN714060 LN714125 |
| Zanclea sp.    | Dipsastrea    | MA012         | Maldives | LN714190 LN714061 LN714126 |
| Zanclea sp.    | Dipsastrea    | MA013         | Maldives | LN714191 LN714062 LN714127 |
| Zanclea sp.    | Dipsastrea    | MA014         | Maldives | LN714192 LN714063 LN714128 |
| Zanclea sp.    | Dipsastrea    | MA015         | Maldives | LN714193 LN714064 LN714129 |
| Zanclea sp.    | Leptoseris    | MA016         | Maldives | LN714194 LN714065 LN714130 |
| Zanclea sp.    | Leptoseris    | MA017         | Maldives | LN714195 LN714066 LN714131 |
| Zanclea sp.    | Leptoseris    | MA018         | Maldives | LN714196 LN714067 LN714132 |
| Zanclea sp.    | Leptoseris    | MA019         | Maldives | LN714197 LN714068 LN714133 |
| Zanclea sp.    | Leptoseris    | MA020         | Maldives | LN714198 LN714069 LN714134 |
| Zanclea sp.    | Leptastrea    | MA021         | Maldives | LN714199 LN714070 LN714135 |
| Zanclea sp.    | Leptastrea    | MA022         | Maldives | LN714200 LN714071 LN714136 |
| Zanclea sp.    | Leptastrea    | MA023         | Maldives | LN714201 LN714072 LN714137 |
| Zanclea sp.    | Leptastrea    | MA024         | Maldives | LN714202 LN714073 LN714138 |
| Zanclea sp.    | Leptastrea    | MA025         | Maldives | LN714203 LN714074 LN714139 |
| Zanclea sp.    | Echinopora    | MA026         | Maldives | LN714204 LN714075 LN714140 |
| Zanclea sp.    | Echinopora    | MA027         | Maldives | LN714205 LN714076 LN714141 |
| Zanclea sp.    | Echinopora    | MA028         | Maldives | LN714206 LN714077 LN714142 |
| Zanclea sp.    | Echinopora    | MA029         | Maldives | LN714207 LN714078 LN714143 |
| Zanclea sp.    | Turbinaria    | MA030         | Maldives | LN714208 LN714079 LN714144 |
| Zanclea sp.    | Turbinaria    | MA031         | Maldives | LN714209 LN714080 LN714145 |
| Zanclea sp.    | Turbinaria    | MA032         | Maldives | LN714210 LN714081 LN714146 |
| Zanclea sp.    | Turbinaria    | MA033         | Maldives | LN714211 LN714082 LN714147 |
| Zanclea sp.    | Turbinaria    | MA034         | Maldives | LN714212 LN714083 LN714148 |
| Zanclea sp.    | Turbinaria    | MA035         | Maldives | LN714213 LN714084 LN714149 |
| Zanclea sp.    | Platygyra     | MA036         | Maldives | LN714214 LN714085 LN714149 |
Table 1. (Continued)

| Species      | Coral host genus | Specimen code | Locality | Genbank accession numbers |
|--------------|------------------|---------------|----------|---------------------------|
| Zanclea sp.  | Platygyra        | MA037         | Maldives | LN714211, LN714086, LN714150 |
| Zanclea sp.  | Platygyra        | MA038         | Maldives | LN714212, LN714087, LN714151 |
| Zanclea sp.  | Platygyra        | MA039         | Maldives | LN714213, LN714088, LN714152 |
| Zanclea sp.  | Platygyra        | MA040         | Maldives | LN714214, LN714089, LN714153 |
| Zanclea sp.  | Symphyllia       | MA041         | Maldives | LN714215, LN714090, LN714154 |
| Zanclea sp.  | Symphyllia       | MA042         | Maldives | LN714216, LN714091, LN714155 |
| Zanclea sp.  | Symphyllia       | MA043         | Maldives | LN714217, LN714092, LN714156 |
| Zanclea sp.  | Symphyllia       | MA044         | Maldives | LN714218, LN714093, LN714157 |
| Zanclea sp.  | Symphyllia       | MA045         | Maldives | LN714219, LN714094, LN714158 |
| Zanclea sp.  | Porites          | MA046         | Maldives | LN714220, LN714095, LN714159 |
| Zanclea sp.  | Porites          | MA047         | Maldives | LN714221, LN714096, LN714160 |
| Zanclea sp.  | Porites          | MA048         | Maldives | LN714222, LN714097, LN714161 |
| Zanclea sp.  | Porites          | MA049         | Maldives | LN714223, LN714098, LN714162 |
| Zanclea sp.  | Montipora        | MA061         | Maldives | LN714232, LN714110, LN714174 |
| Zanclea sp.  | Montipora        | MA062         | Maldives | LN714233, LN714111, LN714175 |
| Zanclea sp.  | Montipora        | MA063         | Maldives | LN714234, LN714112, LN714176 |
| Zanclea sp.  | Montipora        | MA064         | Maldives | LN714235, LN714113, LN714177 |
| Zanclea sp.  | Montipora        | MA065         | Maldives | LN714236, LN714114, LN714178 |
| Zanclea sp.  | XMZS1            | China         | KF962188, KF962532, KF962373 |
| Zanclea sp.  | XMZS2            | China         | KF962189, KF962533, KF962374 |
| Zanclea sp.  | XMZS3            | China         | KF962190, KF962534, KF962375 |
| Zanclea sp.  | XMZS4            | China         | KF962191, KF962535, KF962376 |
| Zanclea sp.  | XMZS5            | China         | KF962192, KF962536, KF962377 |
| Zanclea sp.  | XMZS6            | China         | KF962193, KF962537, KF962378 |
| Zanclea sp.  | XMZS7            | China         | KF962194, KF962538, KF962379 |
| Zanclea sp.  | XMZS8            | China         | KF962195, KF962539, KF962380 |
| Zanclea sp.  | XMZS9            | China         | KF962196, KF962540, KF962381 |
| Zanclea sp.  | XMZS10           | China         | KF962197, KF962541, KF962382 |
| Zanclea costata | MHNG INV26507   | France        | EU876553, EU879951 |
| Zanclea costata | MHNG INV26507   | France        | FN687559  |
| Zanclea giancarloi | MHNG INV26507    | France        | AYS12531  |
| Zanclea giancarloi | MHNG INV26507    | France        | FN687560  |
| Zanclea giancarloi | MHNG INV26507    | France        | FN687561  |
| Zanclea giancarloi | MHNG INV26507    | France        | FN687562  |
| Zanclea sessilis | MHNG INV14438   | France        | AYS12532  |
| Zanclea sessilis | MHNG INV14438   | France        | FN687557  |
| Zanclea sessilis | MHNG INV14438   | Spain         | FN687558  |
| Zanclea prolifera | KUNHM 2793       | Japan         | EU305488, EU272598 |
| Asycoryne ryniensis | KUNHM 2639       | Japan         | EU876552, GQ424289 |
| Cladocoryne floccosa | KUNHM 2639       | Japan         | EU876554, EU272551 |
| Hydractinia vulgaris | Argentina       | EU879941     |
| Hydrocoryne miurenisis | KUNHM 2814   | Japan         | GQ424313  |
| Millepora sp.  | California       | AYS12534     | AYS20801  |

(Continued)
Results

Molecular results

The total genomic DNA of 63 ethanol-fixed Zanclea samples from 13 scleractinian genera was extracted, and three molecular markers were amplified (28S, 16S and COI) for a total number of 183 sequences.

The total alignments of 28S, 16S, and COI datasets were respectively 252, 374, and 647 bp long, while the concatenated set of mitochondrial markers was 1009 bp long. Phylogenetic trees obtained from BI, ML, and MP analyses were similar and, therefore, only Bayesian topologies with significant branch support indicated by Bayesian posterior probability scores, ML bootstrapping supports, and MP bootstrapping supports were shown in Figs 2 and 3 and in S2 and S3 Figs.

The general topologies of 28S and 16S trees (Fig 2 and S2 Fig, respectively) were consistent with previous studies [26, 28]. They confirmed the paraphyly of the Zanclea genus, due to the divergent position of Zanclea prolifera. Furthermore, Zanclea associated with scleractinians and the other Zanclea species not living in association with hard coral are separated by high values of genetic distances, with a mean genetic distance of 6.1 ± 1.5% for 28S and 11.3 ± 1.4% for 16S. The monophyly of Zanclea associated with scleractinians was strongly supported in all the nuclear and mitochondrial phylogeny reconstructions. In the 28S analysis, all our newly obtained sequences clustered in a single lineage together with the other Zanclea associated with scleractinians sequences obtained from previous works [26, 28] but the relationships within this group were unresolved (Fig 2). 16S and COI trees were mostly congruent and their concatenation increased branch support values. Combined mitochondrial 16S and COI phylogenetic tree showed a better resolution of phylogenetic relationships among Zanclea associated with scleractinians and seven well-supported monophyletic lineages were identified (Clades I, II, III, V, VI, VII, and VIII) (Fig 3A, S2 and S3 Figs). 16S tree showed an additional clade (Clade IV) (S2 Fig), due to the presence in the analysis of Acropora-associated Zanclea sp. sequences from Fontana et al. [26], for which no COI sequences are currently available. Almost all of the seven Zanclea clades were genus-specific, except for Clade VIII that was associated with seven
different host genera. Hydroids belonging to Clade I were associated with *Goniastrea* and according to the concatenated analysis they represented the earliest diverging group of *Zanclea* associated with scleractinians (Fig 3A). Other early diverging clades were Clade II and Clade III, which included hydroids symbiotic respectively with *Porites* and *Montipora*. In the 16S tree, Clade III also included a specimen found on *Montipora* from Taiwan by Fontana et al. [26], for which there are no available COI data. *Acropora*-associated hydroids were...
monophyletic and themselves divided in two geographically distinct clades, Clade IV and Clade V, with the latter group corresponding to the nominal species *Z. gallii*. Clade VI was composed by hydroids belonging to the nominal species *Z. sango*, that we found in association with corals of the genus *Pavona*. Finally, *Zanclea* specimens of Clade VII were associated with *Favites*, while Clade VIII consisted of *Zanclea* samples found in association with *Dipsastrea*, *Echinopora*, *Leptastrea*, *Leptoseris*, *Platygyra*, *Symphyllia*, and *Turbinaria*. Within-clade genetic distances were extremely low for both mitochondrial markers being generally 0%, while inter-clade genetic distances were higher for COI rather than for 16S (Tables 2 and 3), with a mean of 6.9 ± 0.6% and 4.4 ± 0.7%, respectively. For example, the genetic distances between *Z. gallii* and *Z. sango* are 7.9 ± 1.1% for COI and 6.1 ± 1.4% for 16S (Tables 2 and 3).
Table 2. Pairwise comparisons and genetic distance. Pairwise comparisons of genetic distance within and between nominal species of *Zanclea* and/or clades of *Zanclea* associated with scleractinians based on the mitochondrial gene 16S.

| Genetic distances | Clade I | Clade II | Clade III | Clade IV | Clade V | Clade VI | Clade VII | Clade VIII | Zanclea sp.* | Z. giancarloi | Z. sessilis | Z. costata | Z. prolifera |
|-------------------|---------|----------|-----------|----------|---------|----------|-----------|------------|-------------|---------------|-------------|------------|------------|-------------|
| Clade I           | 0.0     | (0.0)    |           |          |         |          |           |            |             |               |             |            |            |             |
| Clade II          | 3.2     | (0.9)    | 0.0       | (0.0)    |         |          |           |            |             |               |             |            |            |             |
| Clade III         | 2.9     | (0.9)    | 2.6       | (0.8)    | 0.0     | (0.0)    |           |            |             |               |             |            |            |             |
| Clade IV          | 5.1     | (1.2)    | 4.6       | (1.1)    | 5.5     | (1.2)    | 0.3       | (0.2)      |             |               |             |            |            |             |
| Clade V           | 5.1     | (1.3)    | 4.2       | (1.1)    | 5.1     | (1.2)    | 1.1       | (0.5)      | 0.1         |               |             |            |            |             |
| Clade VI          | 4.1     | (1.1)    | 5.7       | (1.3)    | 5.4     | (1.2)    | 6.5       | (1.3)      | 6.1         | 0.0           |             |            |            |             |
| Clade VII         | 3.8     | (1.1)    | 4.8       | (1.1)    | 3.8     | (1.0)    | 4.6       | (1.1)      | 4.2         | 3.5           | 0.0         |            |            |             |
| Clade VIII        | 4.1     | (1.1)    | 4.4       | (1.1)    | 4.8     | (1.1)    | 4.3       | (1.1)      | 3.9         | 3.2           | 0.9         | 0.0         |            |             |
| Zanclea sp.*      | 10.4    | (1.8)    | 9.4       | (1.7)    | 10.4    | (1.9)    | 11.3      | (1.9)      | 10.8        | 12.4          | 11.0        | 11.4        | 0.0         | (0.0)       |
| Z. giancarloi     | 9.3     | (1.6)    | 8.4       | (1.5)    | 9.0     | (1.6)    | 10.2      | (1.7)      | 10.1        | 11.4          | 9.4         | 9.1         | 1.6         | (0.5)       |
| Z. sessilis       | 9.4     | (1.6)    | 8.3       | (1.5)    | 8.6     | (1.5)    | 10.8      | (1.8)      | 10.7        | 11.3          | 9.9         | 9.6         | 1.6         | (0.4)       |
| Z. costata        | 12.1    | (1.7)    | 12.5      | (1.7)    | 12.2    | (1.7)    | 13.6      | (1.9)      | 13.4        | 14.3          | 12.4        | 12.7        | 1.7         | (1.8)       |
| Z. prolifera      | 15.2    | (2.3)    | 15.2      | (2.2)    | 16.6    | (2.4)    | 18.0      | (2.5)      | 17.4        | 17.3          | 16.2        | 16.6        | 2.2         | (2.2)       |

*Zanclea* sp. sequences from China available in GenBank.

n.c. not calculated

Standard deviations are indicated in brackets.

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Table 3. Pairwise comparisons and genetic distance. Pairwise comparisons of genetic distance within and between species of *Zanclea* and/or clades of *Zanclea* associated with scleractinians based on the mitochondrial gene COI.

| Clade I | Clade II | Clade III | Clade IV | Clade V | Clade VI | Clade VII | Clade VIII | Zanclea sp.* |
|---------|----------|-----------|----------|---------|----------|-----------|------------|--------------|
| Clade I | 0.0      | (0.0)     |          |         |          |           |            |              |
| Clade II| 6.9      | (1.0)     | 0.0      | (0.0)   |         |           |            |              |
| Clade III| 4.9     | (0.9)     | 5.2      | (0.9)   | 0.0      | (0.0)     |           |              |
| Clade V | 9.3      | (1.2)     | 7.5      | (1.1)   | 8.4      | (1.2)     | 0.3       | (0.2)        |
| Clade VI| 8.5      | (1.2)     | 7.6      | (1.1)   | 7.3      | (1.1)     | 7.9       | (1.1)        |
| Clade VII| 8.5     | (1.2)     | 9.2      | (1.3)   | 7.6      | (1.2)     | 9.3       | (1.2)        |
| Clade VIII| 8.0    | (1.1)     | 8.7      | (1.2)   | 8.2      | (1.2)     | 9.5       | (1.3)        |
| Zanclea sp.*| 13.7 | (1.4)    | 16.3     | (1.7)   | 14.9(1.6)| 16.7(1.6)| 13.7(1.4)| 16.2(1.6)    |

*Zanclea* sequences from China available in GenBank.

Standard deviations are indicated in brackets.

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A total of 12 and 10 haplotypes were obtained respectively from 16S and COI sequences of *Zanclea* associated with scleractinians. Median-joining networks for each mitochondrial phylogenetic reconstructions and they are similar between each others. No haplotypes were shared between representatives of two or more clades identified with phylogenetic analyses and, thus, all of the clades were genetically separated from each other. COI haplotypes were separated by an approximate four times higher number of substitutions compared to 16S haplotypes. For example, the only two nominal species of *Zanclea* included in network analyses (*i.e.* Z. *gallii* and *Z. sango*) were separated by 26 substitutions in 16S network and by 81 substitution in COI network.

**Morphological results**

For all the sampled hydroids, the morphology observed was in accordance with the description of the genus *Zanclea* [47]. The polyps arise abundantly from the scleractinian surface, being frequently scattered on the corallite edges or between corallites and have been recorded highly proximal to scleractinian polyps.

As already reported in Montano et al. [28], the morphological characters mainly used to distinguish *Zanclea* species are the organization of the colony (monomorphic or polymorphic), the presence of perisarc that covers the hydrorhiza and hydrocauli, the number of polyp tentacles, the placement of medusa buds on polyps, the cnidome of both polyps and medusae, and the number of cnidophores on the tentacles of medusae. The morphological characters of the clades resulted from the molecular analyses are reported in the Table 4 and in S4 Fig.

**Discussion**

*Zanclea* molecular phylogeny

The results provided in this study currently represent the most comprehensive phylogenetic reconstruction of the genus *Zanclea* with a particular focus on scleractinian-associated species. The broad-based phylogenetic trees obtained with both 28S and 16S molecular markers (Fig 2 and S2 Fig) are consistent with previous molecular studies [26, 60]. These trees essentially depict the genus *Zanclea* as a paraphyletic group within the Zancleida clade [26, 28, 60] due to

| Genetic clade | N° of host genera | Perisarc | Polymorphism | Polyp tentacles | Cnidome | Medusae observation |
|---------------|-------------------|----------|--------------|-----------------|---------|-------------------|
|               |                   |          |              | Oral           | Aboral  | Two-size stenoteles | Macrobasic euryteles |                    |
| I             | 1                 | Yes      | Unknown      | 6              | 25–36   | Yes               | Yes               | No                |
| II            | 1                 | Unknown  | Unknown      | 5–6            | 26–33   | Yes               | No                | No                |
| IIIa,b,c      | 1                 | Yes      | Unknown      | 5–6            | 27–30   | Yes               | No                | No                |
| IVb           | 1                 | Unknown  | Unknown      | Unknown        | Unknown | Unknown           | Unknown           | No                |
| Vc            | 1                 | No       | Yes          | 4–6            | 14–26   | Yes               | No                | Yes               |
| VIc           | 2                 | Yes      | Yes          | 4–6            | 11–22   | Yes               | Yes               | Yes               |
| VIIa          | 1                 | Yes      | Unknown      | 5–6            | 26–30   | Yes               | Yes               | No                |
| VIIb          | 7                 | Yes      | Yes          | 5–7            | 23–35   | Yes               | Yes               | No                |

a present study;  
b Fontana et al. 2012;  
c Montano et al. 2015.
the unresolved position of _Zanclea prolifera_. This species was formally classified in the genus _Zanclea_ even though its polyp stage was unknown [11]. Furthermore, several molecular works have shown that _Z. prolifera_ is more closely related to _Asyncoryne_ spp. than to the other _Zanclea_ species [26, 28, 60]. This genetic evidence is not unexpected considering that _Zanclea_ and _Asyncoryne_ have similar medusae [47, 77]. For this reason, several authors have proposed to move _Z. prolifera_ into the genus _Asyncoryne_ [15, 26, 60, 78], a hypothesis consistent with our 16S phylogenetic tree (S2 Fig).

Both the nuclear and mitochondrial phylogenetic reconstructions resolved _Zanclea_ associated with scleractinians as a monophyletic lineage. As already discussed in Montano et al. [28], the monophyly of _Zanclea_ associated with scleractinians is consistent with the recovery within the genus _Zanclea_ of two distinct groups proposed by Boero et al. [15] mainly based on the occurrence of a monomorphic (the _alba_ group) or polymorphic (the _polymorpha_ group) colony. The latter group counts seven species to date, including three species associated with bryozoans (_Zanclea polymorpha_ Schuchert, 1996, _Z. hirohitoi_ Boero, Bouillon & Gravili 2000, and _Zanclea tipis_ Puce, Cerrano, Boyer, Ferretti & Bavestrello, 2002) and the four currently described _Zanclea_ species associated with scleractinians (_Z. gilii_, _Z. margaritae_, _Z. sango_, and _Z. gallii_). Therefore, the character state “polymorphic colony” could be consistent with the monophyly of _Zanclea_ species associated with scleractinians and with their separation from _Zanclea_ species showing a monomorphic colony. Nevertheless, detailed morphological data are not available for several specimens of _Zanclea_ in symbiosis with scleractinians, and molecular data remain unavailable for most of the nominal species of _Zanclea_, including the polymorphic species associated with bryozoans. Therefore, the evolutionary validity of the distinction between the _alba_ group and the _polymorpha_ group needs to be further addressed in the future with full morphological and molecular analyses of _Zanclea_ species ascribed to the two groups to undertake any formal taxonomic action.

**Genetic diversity of scleractinian-associated _Zanclea_**

In addition to the commonly recommended mitochondrial 16S gene as a DNA barcode for Hydrozoa [40, 45, 79–81], we showed herein that the gene COI allows the recognition of separated hidden lineages in agreement with 16S data, revealing reasonable potential for phylogenetic and evolutionary analyses in the genus _Zanclea_. Indeed, COI turned out to be more variable than 16S, having approximately four times more mutations compared with 16S, despite the analysed portion of COI being bigger than that of 16S (647 bp for COI and 374 bp for 16S). Therefore, the levels of divergence observed within _Zanclea_ associated with the scleractinian group strongly encourage and support the use of both COI and 16S sequences in phylogenetic studies of these hydroids. This conclusion is consistent also with several previous molecular works which successfully used COI gene in order to evaluate the potential presence of cryptic species or intraspecific population subdivision in _Plumularia setacea_ [40], _Obelia geniculata_ [38], and in the genus _Cordylophora_ [81].

According to the mitochondrial phylogenetic trees and haplotype network analyses, all _Zanclea_ specimens associated with scleractinians group together in a cohesive and monophyletic cluster; moreover, they are characterized by considerable genetic diversity (Fig 3A). Indeed, our molecular results indicate that this group is composed of multiple reciprocally well-supported monophyletic lineages (Clades I through VIII) that show a peculiar pattern of host specificity, as discussed in the following paragraph. Two of these seven lineages notably correspond to the nominal species _Z. sango_ (Clade VI) and _Z. gallii_ (Clade V), and the genetic divergence between the two species overlaps the distance values found between all the other molecular clades using both the mitochondrial 16S and COI genes (Tables 2 and 3). Although
we are far from the establishment of an appropriate and widely accepted genetic distance threshold to differentiate hydrozoan species using 16S sequences, Moura et al. [45] proposed a conservative maximum of 2% divergence for intraspecific sequence distance in the Sertulariidae. In our 16S analysis, all the intraclade distances are under this value, while the interclade divergences exceed this conservative threshold in most of the pairwise comparisons. Furthermore the genetic differentiation of 16S locus between our multiple lineages of *Zanclea* (Table 2) is clearly consistent with those calculated between nominal and putative species of the genus *Turritopsis* (3.6%–12.1%) [80] and *Acryptolaria* (up to 3.1%) [42]. Comparable 16S genetic distances revealed the existence of cryptic species within *Cordylophora* (3.3%–6%) [81], *Nemertesia* (up to 4.8%) [43], *Stylactaria* (up to 6%) [46], *Cryptolaria pectinata* (up to 2.2%) [42], and *Lafoea dumosa* (up to 5%) [42, 44].

In conclusion, for both mitochondrial markers, relevant comparisons with previous similar works suggest that the genetic divergence found within *Zanclea* associated with scleractinians might be better explained by assigning independent species status to all molecular clades rather than considering these lineages to be the result of a strong population subdivision. Nevertheless, to discriminate between these two alternative hypotheses, it will be mandatory to corroborate our mitochondrial data with investigations of additional variable nuclear markers and to evaluate the possible presence of morphological features that are clade-diagnostic in the group of *Zanclea* associated with scleractinians.

### Host specificity of *Zanclea* associated with scleractinians

Currently, there is evidence concerning increasing reports of the occurrence of associations between scleractinians and hydroids belonging to the genus *Zanclea* in the coral community [6–8, 26, 27, 29]. This growing number of works likely reflects only a lack of attention about this association in previous decades, due to the small dimensions of hydroids, that have limited their observation. However, the absence of previous data prevents us from excluding a possible recent spread of this association in the reefs of the Indo-Pacific and the Red Sea. Furthermore, our molecular data showed that the genetic diversity within *Zanclea* associated with scleractinians is very high and that there is a multitude of hidden molecular lineages within this group. Boero et al. [15] hypothesized that radiation similar to bryozoan-inhabiting hydroids also occurred in coral-inhabiting hydroids, and the combined morpho-molecular data reported for the recently described species *Z. gallii* [28] as well as the molecular data obtained in the present study, seem to support this hypothesis.

With the exception of the less specialized *Z. alba* (Meyen, 1834), considered a species with characters near to the ancestral state, and *Z. costata*, which is not compulsorily associated with bivalves [15], the genus *Zanclea* usually shows high host specificity [15–19]. The present study suggests the existence of both host-generalist and genus-specific lineages of *Zanclea* associated with scleractinians. In addition to *Z. gallii* living in association with the genus *Acropora* in Maldives, we discovered four well-supported lineages (Clades I, II, III, and VII), each one forming a strict association with a single scleractinian genus. This evidence, together with the close relationship between sequences of *Zanclea* associated with *Montipora* from two geographically separated areas (Maldives and Taiwan), support the hypothesis that *Zanclea* in symbiosis with scleractinians include lineages that settle on scleractinian hosts belonging to a preferred genus, as already suggested by Fontana et al. [26]. However, two host-generalist *Zanclea* lineages were also observed. The first lineage includes *Z. sango*, a nominal species currently known to be associated with the two scleractinian genera *Pavona* and *Psammocora* [7, 28]. In addition, our analysis recovered a second well-supported lineage formed by *Zanclea* specimens symbiotic with seven scleractinian genera (Clade VIII). These two lineages could represent less
specialized and more generalist *Zanclea* lineages living in association with several scleractinians ascribed to different genera.

Concerning morphological traits related to host specificity, Puce et al. [16] noted the importance of the presence or absence of a perisarc around the hydrorhiza. The authors suggested that ancestral species are predicted to be host generalists and characterized by hydrorhiza covered by a perisarc, whereas advanced species that establish specific associations with host species should have lost their perisarc. Although this scenario was already observed between *Z. gallii* and *Z. sango* [28], the morphological results herein obtained reveal the presence of a perisarc covering the hydrorhiza in both host-specific (Cades I, III and VII) and host-generalist (Clades VI and VIII) lineages. This evidence may suggest a less integrated relationship between *Zanclea* belonging to Clades I, III and VII and their host. An alternative hypothesis is that, as the presence of macrobasic euryteles [15], the absence of the perisarc, instead of being a derived character, might be due to independent events of loss and acquisition of the related structure. Despite the absence of some morphological information, the combined characters “perisarc” and “macrobasic euryteles” allow one to distinguish clades I, III and V. In addition, even though the presence of the perisarc is unknown, clade II differs from clade I, and in accord with the possible presence/absence of the perisarc it may be different from clade V or III, respectively. Clades I, VI, VII and VIII share the same state of the characters “perisarc” and “macrobasic euryteles”, but the last three represent a monophyletic clade and their similarities could be related to this condition. The character “polymorphic colony” was frequently unknown owing to the difficulty of noticing the presence of the very contractile dactylozooids. Three of the clades (V, VI, VIII) share polymorphic colonies, but additional investigations are required to determine whether this character is shared between all clades or if it may help to morphologically differentiate them. Moreover, knowledge of the life cycle of the specimens belonging to each clade will provide important information regarding the evolutionary history of *Zanclea* associated with scleractinians.

The available data prevent us from excluding the possibility that some *Zanclea* lineages, as some other cosmopolitan species of hydroids, may be complexes of species [82, 83]. Indeed, nominal species of hydroids known to have a very wide, circumglobal distribution could eventually result in different geographically delimited species [38, 46, 79, 81, 84, 85], sometimes suggesting the existence of cryptic species [40]. At present we can only speculate on the true diversity of *Zanclea* associated with scleractinians because the incomplete set of information currently available makes any discussion inconclusive. In fact, some *Zanclea* species lack complete morphological information, and no DNA sequences are available for the majority of the nominal *Zanclea* species known. Thus, we strongly stress that DNA sequences of already described *Zanclea* species are necessary to clarify the true diversity of the entire genus, and especially of species living in association with scleractinians.

**Conclusion**

The recent literature [6–8, 26, 27, 29] suggests that the *Zanclea*-scleractinians symbiosis is widespread in coral communities of the Indo-Pacific and Red Sea. Although the analysis of species boundaries within the genus *Zanclea* is still far from complete, our results show that the barcoding region of the COI gene is very informative and useful in such scope. Herein, we set a starting point for further investigations, showing high genetic diversity in the *Zanclea*-scleractinian symbiosis and reporting potential hidden lineages both host-specific and host-generalist. Currently, the available morphological data suggest that some identified clades are morphologically different and that the possibility of crypticism between some molecular lineages is observed. Molecular phylogeny is currently revolutionizing the traditional systematics.
in a multitude of marine taxa including Hydrozoa [59, 60, 62]. Therefore, integration between a complete morphological approach that investigates both polyp and medusa stages and a molecular multilocus approach is needed to better clarify the diversity of the $Z$anclea-scleractinian association.

**Supporting Information**

S1 Fig. Map of the study area. A) Maldives; B) Faafu Atoll; C) Magoodhoo Island. (TIF)

S2 Fig. Phylogenetic tree based on the mitochondrial gene 16S inferred by Bayesian inference. The clade support values are $a$ posteriori probabilities ($\geq 0.7$), bootstrap values from Maximum Likelihood ($\geq 70$), and bootstrap values from Maximum Parsimony ($\geq 70$), in this order. (TIF)

S3 Fig. Phylogenetic tree based on the mitochondrial gene COI inferred by Bayesian inference. The clade support values are $a$ posteriori probabilities ($\geq 0.7$), bootstrap values from Maximum Likelihood ($\geq 70$), and bootstrap values from Maximum Parsimony ($\geq 70$), in this order. (TIF)

S4 Fig. Morphological characters of $Z$anclea hydroids associated with scleractinians. A) Gastrozooids and a dactylozooid (arrowhead) emerging from $P$avona varians; B-C) Gastrogonozooid and a blastostyle bearing mature medusa buds on $P$orites sp. and $A$cropora $m$uricata, respectively. D) An extended polyp belonging to clade VIII and growing on $T$urbinaria sp.; E) a contracted dactylozooid belonging to a $Z$anclea $s$ango colony. F-G) Micrographs showing the basal portion of $Z$anclea hydroids associated with $L$eptoseris sp. and $L$eptastrea sp., respectively; the hydrocauli are covered by a transparent perisarc (arrowheads). H) Undischarged two-sized stenoteles; I-J) large and small discharged stenoteles. K-L) Undischarged apotrichous macrobasic eurytele from $Z$anclea $s$ango and a detail of the distal part of the shaft of the same discharged nematocyst. (Scale bars: A-C ~ 0.5 mm; D-G ~ 100 $\mu$m; H-L ~ 5 $\mu$m). (TIFF)

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**Author Contributions**

Conceived and designed the experiments: SM. Performed the experiments: SM DM RA. Analyzed the data: RA DM SP. Contributed reagents/materials/analysis tools: DS PG. Wrote the paper: SM RA DS SP.

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