Evolution and biogeography of the Zanclea-Scleractinia symbiosis

Davide Maggioni¹,² ♦ Roberto Arrigoni³ ♦ Davide Seveso¹,² ♦ Paolo Galli¹,² ♦
Michael L. Berumen⁴ ♦ Vianney Denis⁵ ♦ Bert W. Hoeksema⁶,⁷ ♦
Danwei Huang⁸ ♦ Federica Manca⁹,¹⁰ ♦ Daniela Pica⁹ ♦ Stefania Puce⁹ ♦
James D. Reimer¹⁰,¹¹ ♦ Simone Montano¹,² ♦

Abstract Scleractinian corals provide habitats for a broad variety of cryptofauna, which in turn may contribute to the overall functioning of coral symbiomes. Among these invertebrates, hydrozoans belonging to the genus Zanclea represent an increasingly known and ecologically important group of coral symbionts. In this study, we analysed 321 Zanclea colonies associated with 31 coral genera collected from 11 localities across the Indo-Pacific and Caribbean regions, and used a multi-disciplinary approach to shed light on the evolution and biogeography of the group. Overall, we found high genetic diversity of hydrozoans that spans nine clades corresponding to cryptic or pseudo-cryptic species. All but two clades are associated with one or two coral genera belonging to the Complex clade, whereas the remaining ones are generalists associated with both Complex and Robust corals. Despite the observed specificity patterns, no congruence between Zanclea and coral phylogenies was observed, suggesting a lack of coevolutionary events. Most Zanclea clades have a wide distribution across the Indo-Pacific, including a generalist group extending also into the Caribbean, while two host-specific clades are possibly found exclusively in the Red Sea, confirming the importance of this peripheral

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1 Department of Earth and Environmental Sciences (DISAT),
University of Milano-Bicocca, 20126 Milan, Italy
2 Marine Research and High Education (MaRHE) Center,
University of Milano-Bicocca,
Faafu Magoodhoo Island 12030, Republic of Maldives
3 Department of Biology and Evolution of Marine Organisms (BEOM),
Stazione Zoologica Anton Dohrn Napoli, Villa Comunale, 80121 Naples, Italy
4 Red Sea Research Center, Division of Biological and
Environmental Science and Engineering, King Abdullah
University of Science and Technology (KAUST),
Thuwal 23955-6900, Saudi Arabia
5 Institute of Oceanography, National Taiwan University,
Taipei 10617, Taiwan
6 Taxonomy and Systematics Group, Naturalis Biodiversity
Center, 2300 RA Leiden, The Netherlands
7 Groningen Institute for Evolutionary Life Sciences,
University of Groningen, 9700 CC Groningen, The Netherlands
8 Department of Biological Sciences and Tropical Marine
Science Institute, National University of Singapore,
Singapore 117558, Singapore
9 Department of Life and Environmental Sciences, Polytechnic
University of Marche, 60131 Ancona, Italy
10 Molecular Invertebrate Systematics and Ecology Laboratory,
Graduate School of Engineering and Science, University of
the Ryukyus, Nishihara, Okinawa 903-0213, Japan
11 Tropical Biosphere Research Center, University of the
Ryukyus, Nishihara, Okinawa 903-0213, Japan
region as an endemity hotspot. Ancestral state reconstruction suggests that the most recent common ancestor of all extant coral-associated Zanclea was a specialist species with a perisarc, occurring in what is now known as the Indo-Pacific. Ultimately, a mixture of geography- and host-related diversification processes is likely responsible for the observed enigmatic phylogenetic structure of coral-associated Zanclea.

**Keywords** Ancestral state reconstruction · Coevolution · Cryptic species · Cryptofauna · Hydrozoa · Species delimitation · Symbiome

**Introduction**

Much of marine biodiversity remains unknown (Appeltans et al. 2012) and this is especially true for hyper-diverse ecosystems such as coral reefs, in which only a small fraction of species have been discovered and named (Fisher et al. 2015). However, continuous efforts are being made to increase our knowledge on the inhabitants of coral reef ecosystems, leading to the discovery of new species and associations (Plaisance et al. 2011; Leray and Knowlton 2015; Hoeksema 2017; Maggioni et al. 2020a). Moreover, the application of molecular techniques and integrative taxonomy approaches (Dayrat 2005) has allowed the exploration of previously undetectable diversity (Bickford et al. 2007), and the presence of cryptic taxa (i.e. taxa morphologically indistinguishable) in coral reefs seems to be the norm rather than exception (Rocha et al. 2007). Much of the unknown metazoan diversity in coral reefs occurs likely in the cryptofauna, which is composed of highly diverse but understudied invertebrate taxa inhabiting habitat-forming organisms, in particular living, dead, or fragmented (i.e. coral rubble) scleractinian corals (Reaka-Kudla 1997; Takada et al. 2007; Stella et al. 2011; Enochs and Manzello 2012). Indeed, corals provide a multitude of habitats for these organisms (Stella et al. 2011; Hoeksema et al. 2012, 2017) and form multi-species assemblages known as coral symbiomes (sensu Gates and Ainsworth 2011). Coral-associated organisms find shelter, substrate, and resources on their hosts (Stella et al. 2011; Hoeksema 2017), and are believed to contribute to the overall functioning of coral symbiomes, having roles in nutrient cycling, stress tolerance, and defence (Gates and Ainsworth 2011). The study of the taxonomic composition of these multi-species associations, together with the roles of each coral associate, is therefore crucial to improve our knowledge on the functional biology of corals and coral reefs, and consequently on how species interactions may shape the responses of ecological communities to environmental stresses (Gates and Ainsworth 2011; Stella et al. 2011).

Coral-associated hydrozoans have been found to have a possible beneficial role in coral colony protection against predation and diseases (Montano et al. 2017a). Hydrozoans associated with scleractinian corals belong to the genus Zanclea Gegenbaur, 1856 and live as partially endosymbiotic colonies, with their hydrorhiza (i.e. structure that connects different hydranths in the same colony) extending below coral tissues and only polyps extending outwards (Pantos and Bythell 2010). Coral-associated Zanclea hydroids were first reported by Millard and Bouillon (1974) from Mozambique and later by Boero et al. (2000) with the description of Zanclea gilli Boero, Bouillon & Gravili, 2000, associated with an unidentified scleractinian in Papua New Guinea. Three other species have been described so far, namely Z. margaritae Pantos and Bythell 2010 and Z. gallii Montano, Maggioni & Puce, 2015c, associated with Acropora muricata (Linnaeus, 1758) in Australia and the Maldives, respectively (Panthos and Bythell 2010; Montano et al. 2015a), and Z. sango Hirose and Hirose 2011, hosted by Pavona and Psammocora in Japan (Hirose and Hirose 2011). The association is currently thought to have a wide tropical and subtropical distribution, including the Red Sea (Egypt: Montano et al. 2014; Israel: Pica et al. 2017; Saudi Arabia: Maggioni et al. 2017a), Indian Ocean (Mozambique: Millard and Bouillon 1974; Maldives: Montano et al. 2013), Pacific Ocean (Papua New Guinea: Boero et al. 2000; Australia: Pantos and Bythell 2010; Japan: Hirose and Hirose 2011; Taiwan and Indonesia: Fontana et al. 2012; Fiji: Bonito and McInnis 2019) and the Caribbean Sea (Sint Eustatius: Montano et al. 2017b). Since the first reports of Zanclea associated with unidentified corals, our understanding of host ranges has greatly improved, and currently 61 scleractinian species belonging to 31 genera and nine families have been confirmed as hosts (Montano et al. 2015b,c; Bonito and McInnis 2019).

Previous analyses of the genetic diversity of coral-associated Zanclea from the Maldives and Red Sea revealed the presence of multiple clades that could not be fully distinguished using morphology alone, suggesting the presence of cryptic or pseudo-cryptic species (Montano et al. 2015c; Maggioni et al. 2017a). Indeed, the paucity of ‘traditional’ diagnostic morphological characters is common in most zancleid species (Maggioni et al. 2018), as well as in other hydrozoan species (e.g. Cunha et al. 2017; Miglietta et al. 2019). Some coral-associated genetic groups may be recognised according to their host, as they are specifically associated with a single coral genus (Montano et al. 2015c). However, other molecular clades are generalists, and multiple species of Zanclea may occur on the same coral genus from different localities, as observed for Acropora in the Maldives, Red Sea, and...
Australia which hosts Z. gallii Ia, Z. gallii Ia (sensu Maggioni et al. 2017a), and Z. margaritae, respectively (Pantos and Bythell 2010; Montano et al. 2015c; Maggioni et al. 2017a). Nevertheless, the same coral colony has thus far not been reported to host more than one Zanclea clade.

More recently, the use of novel approaches in hydrozoan taxonomy has shown promising results in discriminating among Zanclea species. For example, Manca et al. (2019) found significant differences in the size of the nematocysts among three coral-associated clades and described the presence of symbiont-related peculiar modifications of the coral skeleton that may have taxonomic value. The latter corresponds to skeletal overgrowths that surround the base of Zanclea polyps, and their size was different among the three investigated clades. Moreover, Maggioni et al. (2020b) found that divergent Zanclea clades associated with Pavona and Goniastrea corals, and with an identical general morphology, show different patterns of green fluorescence in their newly released medusae. Altogether these results suggest that a comprehensive integrative approach may help to shed light on the enigmatic diversity of these symbiotic hydrozoans.

In this study, 321 colonies of Zanclea associated with 31 scleractinian genera from several localities across the entire assumed distributional range of the association were analysed. The integrative study of their diversity, morphology, species boundaries, biogeography, evolution, and relationships with hosts was carried out using genetic information derived from three mitochondrial and four nuclear molecular markers (although nuclear markers appeared to contain little phylogenetic signal in this case).

Materials and methods

Sampling and specimen identification

Sampling was carried out by snorkelling (0–5 m deep) and diving (5–40 m deep) between August 2012 and October 2018 in 11 localities across the Indo-Pacific, Red Sea, and Caribbean Sea (Fig. 1, Table S1). When the presence of Zanclea polyps on scleractinians was recorded in situ (Fig. 2a, b), small fragments of corals and associated hydroids were collected and, when possible, rapidly transferred to bowls filled with seawater, or otherwise directly fixed in 99% ethanol for molecular analyses or 10% formalin for morphological analyses. When laboratory facilities were available, live animals were anaesthetised with menthol crystals in order to let them extend and facilitate further manipulation. Hydroids were carefully detached from their hosts using precision forceps, syringe needles, and micropipettes and subsequently fixed in 99% ethanol or 10% formalin. When Zanclea colonies showed developing medusa buds, they were reared with their host corals in constantly oxygenated bowls filled with seawater, at room temperature, under artificial light, and fed with Artemia nauplii until medusa release occurred (Fig. 2c).

Scleractinian corals were identified at the genus or species level according to Veron et al. (2016) and the most recent taxonomic classification (e.g. Wallace et al. 2007; Benzioni et al. 2010, 2012; Gittenberger et al. 2011; Arrigoni et al. 2014a, b; Huang et al. 2014; Terraneo et al. 2017, 2019; Arrigoni et al. 2020). Zanclea polyps were identified at the genus or species level according to Boero et al. (2000), Pantos and Bythell (2010), Hirose and Hirose (2011), and Montano et al. (2015a). The morphological characteristics of Zanclea colonies were assessed using a Leica EZ4 D stereomicroscope and a Zeiss Axioskop 40 compound microscope using both fresh and formalin-fixed material. Specifically, we focused our attention on the most distinctive characters of Zanclea hydroids, namely the polymorphism of the colony (Fig. 2a, b), the nematocyst types (Fig. 2d–f), and the presence or absence of a perisarc covering the hydrorhiza (Fig. 2g). Regarding the medusa stage (Fig. 2c), we focused on the general morphology and the nematocyst types.

DNA extraction, amplification, sequencing, and dataset assembling

The total genomic DNA was extracted from ethanol-fixed Zanclea using the following protocol: for each sample, a single polyp was washed with MilliQ water, put in 9 µl of milliQ water, and incubated at 90 °C for 10 min; subsequently 1 µl of proteinase K was added and the sample was incubated at 50 °C for 30 min followed by 10 min at 90 °C; finally, each sample was diluted adding 40 µl of milliQ water. Three mitochondrial DNA marker regions were amplified for all samples, whereas four nuclear regions were amplified for a subset of the samples (two or three specimens per mitochondrial clade recovered as a species hypothesis in downstream analyses), using the primers and protocols as in Maggioni et al. (2020c). The amplified mitochondrial molecular markers were: (1) a ~ 600 bp portion of the 16S ribosomal DNA gene (16S rRNA), (2) a ~ 650 bp portion of the cytochrome c oxidase subunit I gene (COX1), and (3) a ~ 650 bp portion of the cytochrome c oxidase subunit III gene (COX3). The amplified nuclear markers were: (1) a ~ 1700 bp portion of the 18S ribosomal DNA gene (18S rRNA), (2) a ~ 1600 bp portion of the 28S ribosomal DNA gene (28S rRNA), (3) a ~ 650 bp portion of the internal transcribed spacer ribosomal region (ITS), and (4) a ~ 350 bp portion of the Histone 3 gene (H3). The success of polymerase chain reactions (PCRs) was checked through 1.5% agarose
electrophoretic runs, and PCR products were purified with Illustra ExoStar (GE Healthcare) and sequenced in both directions with ABI 3730xl DNA Analyzer (Applied Biosystems). The obtained chromatograms were visually checked and corrected with Geneious 6.1.6, primer regions were removed, and protein-coding genes (COX1, COX3, H3) were translated to check for the presence of open reading frames. The sequences were deposited in GenBank.
and accession numbers (MT356227-MT356588, MT356591-MT356612, MT362091-MT362435, and MT363352-MT363597) are listed in Table S1 and S2. Sequences of each marker were aligned using MAFFT 7.110 (Katoh and Standley 2013) with the E-INS-i option, Sequences of each marker were aligned using MAFFT 7.110 (Katoh and Standley 2013) with the E-INS-i option, after adding coral-associated Zanclea sequences generated in previous works (Tables S1, S2). The hydrozoan species Cladocoryne haddoni Kirkpatrick, 1890 and Asyncrone ryniensis Warren, 1908 were selected as outgroups (Maggioni et al. 2017b). All mitochondrial markers were concatenated using Mesquite 3.2 (Maddison and Maddison 2006). Prior to phylogenetic reconstruction and species delimitation analyses, identical sequences of both single-locus and multi-locus (Table S3) mitochondrial datasets were collapsed into representative sequence types using FaBox (Villesen 2007).

Phylogenetic analyses

General statistics of the Zanclea sequences and the variability of the seven DNA regions were calculated with DnaSP 6 (Rozas et al. 2017) (Table S4). Only mitochondrial datasets were employed in downstream analyses, due to extremely low genetic variability of the nuclear markers (Table S4). Partition schemes and models were determined using jModelTest 2 (Darriba et al. 2012) for single-locus datasets (Table S4), and PartitionFinder 2 (Lanfear et al. 2012) for the multi-locus dataset (Table S5), using the Akaike Information Criterion (AIC), the corrected AIC (AICc), and the Bayesian Information Criterion (BIC) as optimality criteria. Phylogenetic inference was performed using Bayesian inference (BI) and maximum likelihood (ML). BI analyses were run using MrBayes 3.2.6 (Ronquist et al. 2012): two independent runs for four Markov chains were conducted for 50 million generations, with trees sampled every 5000th generation, and burn-in set to 25%. Parameter estimates and convergence were checked using Tracer 1.6 (Rambaut et al. 2014). Maximum likelihood analyses were run with RAxML 8.2.9 (Stamatakis 2014) using 1000 bootstrap replicates. Uncalibrated ultrametric Bayesian trees were also reconstructed in BEAST 1.8.2 (Drummond et al. 2012) for further species delimitation analyses, setting a coalescent tree prior and an uncorrelated lognormal relaxed clock. Three replicate analyses were run for 108 million generations, with trees sampled every 10,000th generation, and were combined using LogCombiner 1.8.2 (Drummond et al. 2012) with a burn-in set to 25%. Maximum clade credibility trees were obtained using TreeAnnotator 1.8.2 (Drummond et al. 2012). All analyses were run on the CIPRES server (Miller et al. 2010).

DNA-based species delimitations, genetic distances, and haplotype networks

Distance- and tree-based species delimitation approaches were used to assess the species boundaries in coral-associated Zanclea. All analyses were run separately on the single-locus mitochondrial datasets, after removing the outgroups.

First, the distance-based Automatic Barcode Gap Discovery (ABGD) method was used. The ABDG delimitations (Puillandre et al. 2012) were run on the website abgd web (https://www.abi.snv.jussieu.fr/public/abgd/abgdweb.html) using matrices of genetic distances (p-distance, Kimura 2-Parameter, Jukes Cantor) as inputs. Parameters were set as follows: Pmin = 0.001, Pmax = 0.1, Steps = 100, X = 1.5, Nb bins = 100.

For tree-based methods, different implementations of the Poisson Tree Process (PTP) and Generalised Mixed Yule Coalescence (GMYC) methods were used. Single-threshold (p = 0.001) and multiple-threshold PTP analyses (PTP: Zhang et al. 2013; mPTP: Kapli et al. 2017) were performed on the website mPTP Webservice (https://mptp.h-its.org), using Bayesian trees as inputs. GMYC analyses were run in the R environment (R Core Team 2019) using ultrametric Bayesian trees as inputs. Single-threshold GMYC analyses (Pons et al. 2006) were run using the packages ‘Ape’ (Paradis et al. 2004) and ‘Splits’ (Ezard et al. 2009). Bayesian GMYC analyses (bGMYC) were performed using the package ‘bGMYC’ (Reid and Carstens 2012) on a subset of 100 trees retrieved from the 10,000 posterior trees obtained with each BI analysis.

MEGA X (Kumar et al. 2018) was used to calculate genetic distances within and among species hypotheses retrieved with species delimitation analyses. Genetic distances were calculated as % uncorrected p-distances with 1000 bootstrap replicates for each mitochondrial region. Additionally, to investigate possible geographic structure within the genetic groups, single-locus mitochondrial datasets were used to generate median-joining haplotype networks in PopART 1.7 (Leigh and Bryant 2015), and the results based on the most complete mitochondrial dataset (i.e. 16S rRNA) are shown.

Ancestral state reconstructions

A set of biogeographical, morphological (perisarc and euryteles), and host specificity characters were mapped onto a reduced Zanclea phylogeny (including one specimen for each clade). For the morphological and host specificity characters, two possible states were coded, namely presence-absence, whereas for the distribution four states were coded, namely Indo-Pacific (including the Red Sea), Pacific only, Red Sea only, and Indo-Pacific
(including the Red Sea) + Caribbean Sea. The lack of a
time-calibrated phylogeny and the fact that some lineages
could be present also in other unsampled localities prevented
us from implementing more detailed biogeographic and
speciation models. Stochastic mapping (Huelsenbeck et al.
2003) was used to map probable realisations of the evo-
lution of the considered characters on the coral-associated
Zanclea tree. The analyses were carried out using the
‘make.simmap’ function available in the R package ‘Phy-
density plots (Revell 2013) for morphology and host
state.

Testing for Zanclea-Scleractinia cophylogeny

We tested for cophylogeny between Zanclea and their
scleractinian hosts using a reduced multi-locus phyloge-
netic tree of Zanclea (i.e. including one sample for each
clade) and a multi-locus phylogenetic tree including all
scleractinian genera known to host Zanclea hydroids. The
scleractinian dataset was assembled via mining sequences
from GenBank for four loci, namely COXI, ITS, 28S
rRNA, and H3 (Table S6), using the corallimorpharian
Ricordea florida Duchassaing & Michelotti, 1860 as out-
group. Phylogenetic reconstructions were performed using
BEAST 1.8.2 as described above.

Cophylogeny analyses were performed using two
methods: the global-fit PACo 1.2 (Balbuena et al. 2013;
Hutchinson et al. 2017) and the event-based Jane 4 (Conow
et al. 2010). PACo (Procrustes approach to co-phyloge-
netics) assesses the global-fit of symbiont phylogeny onto
the hosts and evaluates the contribution of each individual
host-symbiont association to the global-fit. The overall
phylogenetic congruence was tested with 10⁴ permutations
of the coral-Zanclea association matrix and the contribu-
tion of each individual association was assessed by taxon
jackknifing. PACo analyses were run in the R environment
(R CORE Team 2019). Jane uses an event-based approach
that takes into consideration various events, namely
cospeciation, intra-host diversification (duplication), host
switch, failure to diverge, and loss, to which it assigns
different costs. The optimal (minimum) costs found for the
host-symbiont dataset are compared against randomised
datasets. Jane analysis was run using default cost settings,
generations = 100, population size = 500, and sample
size = 100. Finally, Zanclea-scleractinians interactions
were visualised with TreeMap3 (Charleston and Robertson
2002), using the untangling function to improve the layout.

Results

Updated distribution and host range

A total of 321 Zanclea colonies were examined, 73 of
which were already partially analysed in previous studies,
and the rest newly examined in this study. Hydroids were
obtained from 11 tropical and sub-tropical localities
(Fig. 1, Table S1), with most samples collected in the
Maldive (n = 128), Red Sea (n = 117), Singapore
(n = 30), and Taiwan (n = 23). From other localities we
obtained a lower number of samples (total n = 23). Nota-
ably, despite the Caribbean Sea being explored in multiple
surveys at different localities (Panama, Bonaire, Curacao,
and Sint Eustatius) with a high number of dives and
snorkelling activities, only two Zanclea colonies were
found in Sint Eustatius. The current distributional range of
coral-associated Zanclea covers the Red Sea, Indian
Ocean, Western Pacific Ocean, and the Caribbean Sea. In
this work we succeeded in obtaining samples from most
previously reported localities, with the exception of
Mozambique, Papua New Guinea, and Fiji, where we did
not perform any field work. Specimens in this work from
Singapore, Thailand, and Madagascar represent new geo-
graphic records for Zanclea (Fig. 1).

Altogether, the collected Zanclea colonies were associ-
ted with 31 coral genera and 10 coral families (Table S1).
These records were added to previously reported associa-
tions (Table S7), resulting in a total number of at least 90
host-coral species for Zanclea, belonging to 34 genera and
10 families. With this work, we also report new host
records, including 32 at the species level, three at the genus
level (Acanthastrea, Astrea, Coscinaraeae), and one at the
family level (Coscinaraeidae) (Table S7).

Overall phylogenetic diversity

Genomic DNA was successfully extracted from all speci-
mens and the seven molecular markers were amplified and
sequenced with high success (99%, n = 981), for a total of
972 newly obtained sequences. Nuclear markers were
sequenced for a subset of the dataset and showed extremely
low levels of variability (e.g. % of variable sites ranging
from 0.1 to 2.5%), whereas mitochondrial markers showed
higher genetic variability (Table S4). jModelTest and
PartitionFinder found different best-fit evolutionary models
and partitions according to the information criteria used
(Table S4, S5). All downstream analyses were repeated for
each model and partition, with no resulting substantial
difference in tree topology and support values. Likewise, BI and ML analyses of single- and multi-locus mitochondrial datasets resulted in similar trees (Figs S1, S2), and the multi-locus Bayesian tree is shown in Fig. 3. Specifically, ML and BI trees showed concordant topologies and differences among single-locus trees based on different loci were mostly related to the relationships among clades M1, P1, and G1 and the position of clade P2 (Fig. S1). These uncertainties were also reflected in the low support values in the ML multi-locus tree at these nodes (Fig. S2).

Coral-associated Zanclea were confirmed as a fully supported monophyletic group showing considerable genetic diversification, with nine highly or fully supported clades identified in all phylogenetic analyses (Fig. 3, Figs. S1, S2). The nine groups were named with alphanumeric codes, with letters indicating host-corals, as follows: A1 (Acropora and Isopora), A2 (Acropora), G1 and G2 (generalists), M1 and M2 (Montipora), P1 and P2 (Porites), and Pa (Pavona). Seven out of the nine clades have already been reported in previous works (Montano et al. 2015c; Maggioni et al. 2017a), whereas the remaining two clades (M2 and P2) were found for the first time in this study. The internal nodes were in some cases well supported, but the relationships among lineages G1, M1, P1 and P2 were not fully resolved, as shown by the low BI posterior probabilities and ML bootstrap values.

General descriptions of the clades

The nine Zanclea clades showed contrasting patterns of host-specificity, as both generalist and specialist (at genus level) groups were found (Fig. 3). The two generalist clades, G1 and G2, are associated with 12 and 24 scleractinian coral genera belonging to six and nine families, respectively. These two clades showed a partial overlap of host-coral genera, as nine out of the 12 scleractinian genera associated with G1 also hosted G2, to the exception of Acanthastrea, Astrea and Gardineroseris. The remaining seven Zanclea clades were coral genus-specific in their associations, given our sampling. Clade A1 corresponds to Zanclea gallii and despite being associated with two genera, Acropora and Isopora, we consider this clade as genus-specific due to the close relationship between the two acroporid genera (Wallace et al. 2007). Australian Acropora-associated specimens were collected relatively close to the type locality of the formally described species Zanclea margaritae, and sequences of these samples also fell in this clade. The sister taxon of clade A1 was A2, which was associated with Acropora and found exclusively in the Red Sea, and previously designated as Z. gallii IIa (Maggioni et al. 2017a). The Pavona-associated clade Pa corresponds to Zanclea sango, and this was further supported by our specimens being collected from the type locality (Okinawa, Japan). Zanclea sango was originally described in association with Pavona (= holotype) and Psammocora contigua (Esper, 1794) (= paratypes) (Hirose and Hirose 2011). However, the only specimen collected from Okinawa in this study associated with P. contigua was found to belong to clade G1. Porites corals hosted clade P1, distributed in the Indo-Pacific and Red Sea, and the newly discovered clade P2, collected only from Pacific localities. Finally, Montipora corals hosted a clade found in the Red Sea and Indo-Pacific (M1) and another one exclusive to the Red Sea (M2).

DNA-based species delimitations and genetic distances

Species delimitation analyses consistently suggested that the nine Zanclea phylogenetic clades correspond to nine putative species (Fig. 4a). The distance-based method ABGD identified the nine species hypothesis as most likely in all analyses, whereas the tree-based methods found in some cases a further subdivision, especially concerning clades Pa and G2. However, the vast majority of the analyses agreed in identifying nine putative species. The analysis of intra- and inter-clade genetic distances based on the three mitochondrial markers (Table S8) revealed moderate to high divergence among the Zanclea clades. Specifically, the average inter-clade distances ranged from 3.0 ± 0.6% to 5.6 ± 0.9% for 16S rRNA dataset, from 4.9 ± 0.8% to 9.1 ± 0.9% for the COX1 dataset, and from 7.8 ± 1.0% to 10.8 ± 1.0% for the COX3 dataset. Intra-clade distances were generally low for the 16S rRNA (mean value 0.5 ± 0.1%) and slightly higher for the other, more variable, mitochondrial regions (0.8 ± 0.2% for COX1 and 1.1 ± 0.2% for COX3).

Morphology

The morphology of all polyps included in molecular analyses was investigated in order to detect the presence of perisarc, euryteles, and polymorphic colonies; the morphological characteristics of each clade are summarised in Table 1. Morphology alone was not sufficient to distinguish among most of the clades, according to the morphological characters sampled in this work, although the addition of other characters, such as nematocyst size variation and fluorescence patterns, may reveal fine-scale differences in future works. Specifically, clades G1, G2, and Pa have identical morphologies (perisarc, euryteles, polymorphic colonies), as do clades A1 and A2 (no perisarc, no euryteles), and P1 and P2 (perisarc, no euryteles). Both M1 and M2 clades have morphologies similar to those of G1, G2, and Pa, but euryteles were observed to be very rare. When a perisarc was present, micro-alterations of the host
skeleton were generally found (Fig. 2h). Newly released medusae (Fig. 2c) were observed for five clades (Table 1) and were almost identical, with the only difference being the presence of euryteles in medusae belonging to clades in which polyps possessed euryteles. According to ancestral state reconstructions, the most recent common ancestor of all extant coral-associated Zanclea had a perisarc, and this structure was lost only once, in the common ancestor of the two extant Acropora-associated genetic groups (A1 and A2) (Fig. 5a). The latter two clades also lost the presence of euryteles in their cnidome, and the same event also happened independently in the two Porites-associated groups (P1 and P2) (Fig. 5b).

Biogeographical patterns

The intra-clade genetic diversity followed geographical patterns only for the Acropora-associated hydroids, showing similar results for each of the mitochondrial region analysed (Fig. 4b–g, Fig. S5). In this clade, different localities never shared identical haplotypes and Maldivian, Pacific, and Red Sea populations could be easily distinguished in clade A1 (Fig. 4d, Fig. S5). Additionally, clade A2, which is found exclusively in the Red Sea, seemed to be more closely related to the A1 Red Sea population rather than to other Indo-Pacific populations. In contrast, all other clades had haplotypes shared by multiple localities and no clear geographic structure was identifiable. Overall, most clades have a wide distribution, including the Indo-Pacific and Red Sea.

According to the biogeographic ancestral state reconstruction analyses (Fig. 5d), only a few terminal branches had different distributions, whereas the most recent common ancestor of coral-associated Zanclea showed uniform and wide distributional ranges across the Indo-Pacific.

Fig. 3 Multi-locus (16S rRNA, COX1, COX3) phylogenetic hypothesis of coral-associated Zanclea. Alpha-numeric codes at terminal nodes indicate the representative sequence types (as shown in Table S3), followed by the number of the collapsed sequences in brackets, and the sampling locality, as coded in the legend. Numbers at nodes represent Bayesian posterior probabilities (> 0.9) and maximum likelihood bootstrap values (> 90), respectively, whereas asterisks denote maximal statistical support (1/100). Each recovered clade is highlighted with a different colour, following Montano et al. (2015c) and with a code, as detailed in the ‘Results’ section. On the right side of the tree, the coral genera associated with each clade are shown, divided by family and with family names coded as in the legend.

Fig. 4 a Summary cladogram showing the DNA-based species delimitation results. Grey cells denote that the clade has been successfully delimited as a putative species, yellow cells indicate a further subdivision in one or more clades. b–g 16S rRNA medium-joining networks of Zanclea clades b G2 (generalist), c Pa (Pavona), d A1 + A2 (Acropora, Isopora; dashed lines delimit the two clades) e P1 (Porites), f M1 (Montipora), g G1 (generalist). Colours denote different provenience of the haplotype, as shown in the legend. n. s. not sequenced.
Specifically, two genetic groups appeared to have originated in the Red Sea (A2 and M2), one is currently found only in Pacific localities (P2), and only the generalist clade, G2, is also found in the Caribbean Sea.

**Relationships with the hosts and coevolutionary analyses**

Based on the proposed ancestral state reconstruction, the association between *Zanclea* and scleractinians arose as a host-specific relationship, and generalism emerged independently in the two extant generalist clades (Fig. 5c). The tanglegram (Fig. 6) shows a map of 44 *Zanclea*-coral associations. All specialist groups were associated with corals belonging to the Complex clade, whereas the generalist clades were associated with both Complex and Robust genera (Fig. 6). The same host coral can be involved in associations with multiple *Zanclea* clades (Table 1, Fig. 6). However, this pattern was, in most cases, not observed at the same locality, with the exceptions listed in Table 2, and the same coral colony was never observed to host multiple *Zanclea* clades. The PACo analyses revealed no significant congruence between *Zanclea* and coral phylogenies (global goodness-of-fit statistic \( m^2 = 1.362, p = 0.147 \)). Despite the absence of significance, different links between generalist clades and their hosts contributed relatively little to the \( m^2 \) (Fig. S4), and therefore these may represent coevolutionary links. The Jane analysis resulted in 71 putative evolutionary scenarios, namely zero co-speciations, eight duplications, five host switches, 23 losses, and 35 failures to diverge (Fig. S5).

**Discussion**

**Molecular phylogenetics of coral-associated *Zanclea***

The results provided in this study represent the most comprehensive phylogenetic assessment of coral-associated *Zanclea* to date, including specimens associated with almost all known hosts (31 of 34 reported host genera) and from reported localities. According to the mitochondrial phylogenetic tree, all *Zanclea* specimens associated with scleractinians clustered together in a fully supported monophyletic group. Moreover, *Zanclea* is characterised by considerable genetic diversity that is not reflected in the morphological characters we sampled. Indeed, molecular phylogenetic, species delimitation, and genetic distance analyses revealed the presence of nine well-supported and divergent clades, whereas only three morphotypes were distinguishable: the *Z. sango* type, with a perisarc and euryteles (Pa, G1, G2, M1, M2); the *Z. gallii* type, devoid of a perisarc and euryteles (A1, A2); and a third type with a perisarc but no euryteles (P1, P2). Therefore, in all three morphotypes, cryptic or pseudo-cryptic species are present, and except for the *Z. gallii* type, these morphospecies are not monophyletic. These results are in line with recent DNA-based works showing that the presence of cryptic species is a rather common phenomenon both in Hydrozoa (e.g. Postaire et al. 2016; Vaga et al. 2020) and other cnidarian classes (e.g. Dawson and Jacobs 2001; Bartošová and Fiala 2011; Arrigoni et al. 2019). Hopefully, further detailed morphological assessments of these cryptic groups, including for instance the statistical treatment of morphometric data (Manca et al. 2019) and the observation of green fluorescence patterns

### Table 1 Summary of the host-specificity, distributional, and morphological data for the nine *Zanclea* clades

| Clade | No. of hosts | Host overlap | Distribution | Colony polymorphism | Perisarc (oral + aboral) | Polyp two-sized stenoteles | Polyp euryteles | Newly released medusa observation |
|-------|--------------|--------------|--------------|---------------------|-------------------------|---------------------------|-----------------|----------------------------------|
| A1    | 2            | A2           | Indo-Pacific, Red Sea | Yes                  | No                      | 4–6 + 14–26               | Yes             | No                               |
| A2    | 1            | A1           | Red Sea       | n. o.               | No                      | 5 + 15–25                 | Yes             | No                               |
| G1    | 12           | G2           | Indo-Pacific, Red Sea | Yes                  | Yes                     | 4–7 + 11–39               | Yes             | Yes                              |
| G2    | 25           | G1, Pa       | Indo-Pacific, Red Sea, Caribbean | Yes                  | Yes                     | 4 + 22                    | Yes             | Yes                              |
| M1    | 1            | M2           | Indo-Pacific, Red Sea | n. o.               | Yes                     | 5–7 + 16–64               | Yes             | Yes*                            |
| M2    | 1            | M1           | Red Sea       | n. o.               | Yes                     | 4–8 + 13–38               | Yes             | Yes*                            |
| P1    | 1            | P2           | Indo-Pacific, Red Sea | n. o.               | Yes                     | 4–6 + 28–37               | Yes             | No                               |
| P2    | 1            | P1           | Pacific       | Yes                  | Yes                     | 4 + 32                    | Yes             | No                               |
| Pa    | 1            | G2           | Indo-Pacific, Red Sea | Yes                  | Yes                     | 4–7 + 11–25               | Yes             | Yes                              |

n. o. not observed, *very rare
(Maggioni et al. 2020b), will eventually allow us to find consistent morphological differences and formally describe the species.

Interestingly, the nuclear region sequences we analysed were extremely conserved. By contrast, mitochondrial sequence divergence was high and comparable to what has been previously observed for taxa that are evolutionarily close to *Zanclea*, such as *Millepora* spp. (Arrigoni et al. 2018). Cnidarian mitochondrial DNA has been reported to have slow rates of nucleotide substitution compared to nuclear DNA in Anthozoa (Shearer et al. 2002), but multiple studies have documented faster rates in medusozoan lineages, including hydrozoans (e.g. Govindarajan et al. 2005; Huang et al. 2008), possibly due to mitochondrial DNA linearization and loss of DNA repair genes (Shearer et al. 2002; Hellberg 2006). The observed *Zanclea* mitonuclear discordance may be due to incomplete lineage sorting (Toews and Brelsford 2012) as result of a recent origin coupled with higher evolutionary rates of mitochondrial DNA. Alternative possible explanations may be related to phylogenetic inadequacy of nuclear markers, which are nevertheless able to distinguish other closely related cryptic hydrozoans (Maggioni et al. 2016; Montano et al. 2017c), or introgressive hybridisation (Toews and Brelsford 2012). Whatever the cause of the genetic patterns observed in this study may be, the mitochondrial clades
show, at least in some cases, morphological variation and peculiar host specificity patterns that suggest the presence of evolutionarily independent units, corresponding to different species.

Translating *Zanclea* clades into the known morphospecies

With our analyses we could confidently identify the clade Pa as *Z. sango*, due to the inclusion of specimens associated with *Pavona* from the locality of the holotype, Okinawa. Hirose and Hirose (2011) referred to hydroids associated with *P. contigua* in Japan as belonging to *Z. sango*, due to their completely overlapping morphologies, but here we demonstrate that *P. contigua*-associated specimens likely actually belong to clade G1 in the same locality. However, we cannot disregard that Pa and G1 are associated with both genera in Japan.

The clades G1, G2, M1, and M2 are morphologically identical to *Z. sango*, based on the characters analysed in this study, even if genetically divergent. Euryteles were previously not reported in the *Montipora*-associated clade M1 (Montano et al. 2015c), but they were found in the present work, even if very rare, perhaps the reason why they were not detected in previous works.

*Zanclea margaritae* and *Z. gallii* were described from Australia and the Maldives, respectively, and are very similar from a morphological and host-specificity point of view. Both species are associated with *Acropora* and the only morphological differences are in the cnidome, with *Z. margaritae* possessing mastigophores and isorhizas (Pantos and Bythell 2010). In this study, we analysed several *Acropora*-associated *Zanclea* colonies from many localities, including Australia, but we did not find evidence of these nematocyst types in any investigated polyps, therefore identifying all specimens as *Z. gallii*. However, in some cases we observed *Acropora* nematocysts attached to hydranths, including mastigophores and isorhizas similar in shape and size to those characterised by Pantos and Bythell (2010) as part of the *Z. margaritae* cnidome. Additionally, Pantos and Bythell (2010) found isorhizae in a single hydranth and analysed newly released medusae for three-four hours post-release, increasing the likelihood that mastigophores and isorhizae may come from coral tissues. Therefore, if the mastigophores and isorhizae of *Z. margaritae* are of coral origin, it is possible that *Z. gallii* is a junior synonym of *Z. margaritae*, but further
morphological studies are needed to address this issue, since no type material for Z. margaritae was analysed in this work.

The combination of morphological features found in the third morphotype (perisarc, no euryteles) and consisting of clades P1 and P2 in the phylogenetic tree, does not fit with any of the formally described coral-associated Zanclea species. Nevertheless, description of these clades is currently not possible as specimens of this morphotype cluster in two divergent groups that are not sister taxa. Moreover, we currently lack information on their medusa stage.

Zanclea gilii is a polymorphic species with euryteles and no reported perisarc (Boero et al. 2000). Zanclea gilii morphologies were not observed in any of the samples analysed in this work. The presence of a perisarc is sometimes difficult to spot without histological examination due to the high levels of Zanclea-host integration (Manca et al. 2019), and if the perisarc has been overlooked in Z. gilii, this species would then have a morphology identical to Z. sango. However, the lack of genetic material and information on the host-corals of Z. gilii prevents any further conclusions for now.

Biogeography of the association

Overall, the association between Zanclea and scleractinians has a wide distribution and most clades are found across multiple localities in the Indo-Pacific and Red Sea, generally without clear geographic structure. The Zanclea and scleractinian association seems to have originated in the Indo-Pacific, and only a few terminal branches show variations in their distribution, including two groups possibly endemic of the Red Sea (A2 and M2), a clade currently found only in Pacific localities (P2), and a fourth one that is also present in the Atlantic Ocean (G2). For instance, Z. gallii (clade A1) is the only clade that shows a geographic structure, with distinct Indian, Pacific, and Red Sea populations, and the Red Sea-endemic clade A2 may have originated from Z. gallii populations in the Red Sea. Indeed, the Red Sea is considered a biodiversity and endemivity hotspot (Hughes et al. 2002) where new biodiversity is generated and eventually exported (Bowen et al. 2013, 2016), and the high number of endemics in the region is known to have multiple origins, due to many recent and past isolating barriers (DiBattista et al. 2016). The analyses of Zanclea specimens from neighbouring areas together with a temporal characterisation of Zanclea evolution would greatly help in understanding the origin of the Red Sea endemic clades.

The most widespread clade (G2) is found in the Indo-Pacific, Red Sea and Caribbean regions, and is also associated with the highest number of coral genera. Its extensive generalist habits could have promoted its spread into multiple localities, facilitating the establishment of associations with new hosts, as demonstrated by a widespread coral-associated crab that was recently discovered in Hawai‘i (Hoeksema et al. 2018). However, the lack of fossil records to time-calibrate the Zanclea radiation and the likely wider Zanclea distribution and diversity prevent us from further inferring historical biogeographic patterns. Therefore, the biogeographic analysis presented here should be interpreted with caution until additional sampling in the Pacific and Atlantic oceans is carried out to achieve a time-calibrated analysis of Zanclea.

Relationships among Zanclea clades and their scleractinian hosts

The updated list of scleractinian hosts reveals that Zanclea is able to establish associations with a diverse array of coral species and genera that show multifaceted morphological and ecological characteristics. Most observations were limited to shallow depths (e.g. Montano et al. 2017a), but coral-associated Zanclea was also reported from depths up to 41 m deep (Montano et al. 2014, 2017b; this study) (Table S1) and further explorations may therefore reveal other host-coral species living in the mesophotic. Similarly, no azooxanthellate scleractinians were reported as hosts of Zanclea, but this could be due to limited observation efforts for such corals.

All but two Zanclea clades are involved in specific associations and this specificity is maintained across distant localities. Contrarily, the generalist Zanclea clades are hosted by a large number of phylogenetically distant coral genera. Zanclea is known to establish symbiotic relationships not only with corals, but also with other sessile invertebrates (Boero et al. 2000), and it has been suggested that ancestral species were generalists without adaptations to their host or substrate, such as the loss of the perisarc (Puce et al. 2002).

However, the present study demonstrates that the association between Zanclea and scleractinians likely arose in combination with host specificity, and generalism has been later achieved twice in the evolution of this taxon. Regarding the hypothesised derived host-related loss of perisarc, this feature is found only in Acropora-associated clades, whereas all the other extant generalist and specialist clades, and also their most recent common ancestors, have their hydrorhiza covered by a perisarc. The loss of the perisarc is therefore better explained as an adaptive modification due to specific association with Acropora (and Isopora) spp. rather than a general derived feature of specialist clades.

An interesting aspect common to all specialist clades is the association with corals belonging only to the Complex clade, whereas generalists are hosted indiscriminately by
both Complex and Robust corals. At the moment, it is difficult to suggest possible explanations for this pattern relying on differences between Complex and Robust clades, since the two scleractinian groups are mostly based on molecular and embryogenetic data rather than on morphological, biological, or ecological criteria (Romano and Palumbi 1996; Okubo et al. 2013, 2016; Okubo 2016).

Other than this peculiar pattern, there is limited congruence between the Zanclea and coral phylogenies, refuting the hypothesis of general coevolutionary patterns. This could be due to the very likely fact that the diversification of extant coral genera occurred much earlier than the radiation of extant coral-associated Zanclea. One of the main mechanisms underlying Zanclea radiation, acting both alone and in conjunction with geography-related processes, may be host-switching to exploit new resources and subsequent specialisation of the association and reproductive isolation, as shown in other coral-associated taxa (see review by Potkamp and Fransen 2018). This scenario may apply especially for specialist Zanclea clades. However, a further complication is the presence of host overlap among different clades, which is for instance commonly observed among closely related crustacean symbiont taxa inhabiting the same mushroom coral species (van der Meij et al. 2015; Ivanenko et al. 2018; Rauch et al. 2019).

In previous works, no coral genera were found to host more than one Zanclea clade, with the only exception being Acropora spp. (Montano et al. 2015c; Maggioni et al. 2017a), maybe because it is also the most speciose scleractinian genus of reef-dwelling corals (Hoeksema and Cairns 2020). The large-scale sampling conducted in this work indicates that host overlap at genus level is a common phenomenon, both when examining the entire distributional range of clades, and also at the local scale. Therefore, according to the currently available data on the group, the puzzling genetic structure of coral-associated Zanclea seems to be ultimately due to a mixture of relatively recent geography- and host-related processes, in which allopatric, sympatric, and ecological diversifications have played important roles in shaping the current Zanclea diversity.

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