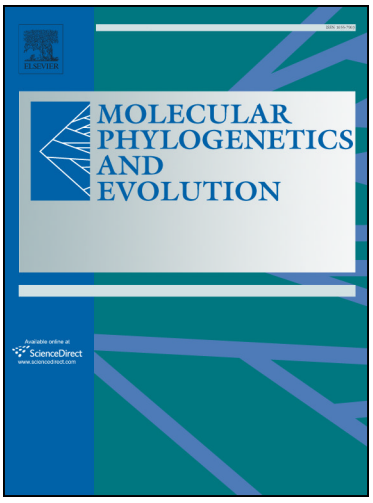

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Lack of host specificity of copepod crustaceans associated with mushroom corals in the Red Sea

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Abstract

The radiation of symbiotic copepods (Crustacea: Copepoda) living in association with stony corals (Cnidaria: Scleractinia) is considered host-specific and linked to the phylogenetic diversification of their hosts. However, symbiotic copepods are poorly investigated, occurrence records are mostly anecdotal, and no explicit analysis exists regarding their relationship with the hosts. Here, we analysed the occurrence of symbiotic copepods on different co-occurring and phylogenetically closely related scleractinian corals. We used an innovative approach of DNA extraction from single microscopic specimens that preserves the shape of the organisms for integrative morphological studies. The rationale of the study involved: (i) sampling of mushroom corals (Fungiidae) belonging to 13 species and eight genera on different reefs along the Saudi coastline in the Red Sea, (ii) extraction of all the associated copepods, (iii) morphological screening and identification of copepod species, (iv) use of DNA taxonomy on mitochondrial and nuclear markers to determine species boundaries for morphologically unknown copepod species, (v) reconstruction of phylogenies to understand their evolutionary relationships, and (vi) analysis of the ecological drivers of the occurrence, diversity and host specificity of the copepods. The seven species of coral-associated copepods, all new to science, did not show any statistically significant evidence of host-specificity or other pattern of ecological association. We thus suggest that, contrary to most assumptions and previous anecdotal evidence on this coral-copepod host-symbiont system, the association between copepods and their host corals is rather labile, not strict, and not phylogenetically constrained, changing our perception on evolutionary patterns and processes in symbiotic copepods.

Keywords: Copepoda, coral reefs, epibiosis, host relationship, integrative taxonomy, phylogenetic signal, symbiosis

1. Introduction

Stony corals (Cnidaria: Scleractinia) are known to host a large diversity of associated fauna, with at least 860 species of symbionts; more than a third (360+) of them are copepods, which can be found, mostly as ectosymbionts, on about 150 host coral species, mainly in the Indo-Pacific and the West Atlantic (Humes, 1979, 1985, 1991; Stella et al., 2011; Cheng et al., 2016; Hoeksema et al., 2017). Although symbiotic copepods have been reported from many species of stony corals, the understanding of their diversity and distribution is highly incomplete and geographically limited (Humes, 1985, 1994; Ho 2001). A single scleractinian coral can host thousands of copepod individuals of up to nine species (Humes, 1985; Cheng et al., 2016). Copepods living on stony corals are represented by a group of families of the orders Poecilostomatoida (288 species), Siphonostomatoida (68 species), Cyclopoida (three species), and Harpacticoida (seven species) (Cheng et al., 2016).

Symbiotic copepods are regularly found strictly associated to the external surface of the corals, but can be present to a lesser extent also in galls, polyps, and intestinal cavities (Stock, 1975; Humes, 1985; Dojiri, 1988; Kim and Yamashiro, 2007; Ivanenko at al., 2014). The biology of most of the symbiotic copepods and their functional relationship with stony corals is unknown; however, the diversity of their body shape (cyclopiform, laterally or dorsoventrally flattened, fossiliform, and vermiform) and their feeding apparatus (cutting and sucking) indicate a variety

of possible relationships with their host corals. Observations of living corals showed that some ectosymbiotic copepods, such as poecilostomatoid xarifids can cause polyps to open their mouth or to modify the shape of their corallite (Cheng and Dai, 2009). Neither pathogenicity of copepods nor any role in transferring pathogenic agents to corals has been recorded to date. However, there are reports of significant destruction of aquarium stony corals for some species of *Acropora* caused by the copepod pest *Tegastes acroporanus* Humes, 1981, described living on *A. florida* (Dana, 1846) from the Marshall island (Humes, 1981; Sweet et al., 2012; Barrett et al., 2016). This suggests that copepods can influence their host corals in the wild, and the role may become even more relevant under detrimental conditions of bleaching or other stresses for corals.

Taxonomic reports on copepod relationships with stony corals show that most of the copepod species are found only on a single species of host coral (70% of the 363 described scleractinian-associate copepod species) or only on coral species of the same genus (17% of scleractinian -associated copepod species); only about 5% of the copepod species are found on stony corals of two or more families (Cheng et al., 2016). No coral-associated copepod has ever been found on other invertebrate hosts. Thus, the literature shows remarkable specificity for host species or host genera for most copepods associated with stony corals. Unfortunately, understanding of host specificity is based on very limited samples for each copepod species (Cheng et al., 2016) and the strict association may be a biased result of a lack of data. No detailed screening of the whole community of associated copepods for several phylogenetically related co-occurring species of stony corals has been performed until now. The aim of the present study is to explicitly test for the degree of host specificity in the copepods associated with stony corals, in order to provide a more reliable picture of the role and the strength of the symbiotic relationship.

As a host model group, we selected a monophyletic group of stony corals that is well known for its associated symbiotic copepods, i.e., the scleractinian family Fungiidae (Hoeksema et al., 2012), popularly known as mushroom corals, and for which the phylogeny is well studied (Wells, 1966; Cairns, 1984; Hoeksema, 1989, 1991, 1993; Gittenberger et al., 2011; Benzoni et al., 2012; Oku et al., 2017). One specific trait shown by the majority of fungiids is a free-living ('anthocyathus') phase, in which the whole coral becomes detached from its substrate and therefore is able to grow soft tissue all around its skeleton (Hoeksema and Yeemin, 2011). Due to the overall shape of mushroom corals, their lower side offers shelter to various species of invertebrates that prefer to hide, at least during daylight (Hoeksema and Fransen, 2011; Gittenberger and Hoeksema, 2013; Hoeksema et al., 2013a, 2013b; Alamaru et al., 2016). However, this shelter can become temporarily lost when the corals become buried or overturned (Bongaerts et al., 2012; Hoeksema and Bongaerts, 2016). Individuals of other associated symbiotic species, which may include many other invertebrates and various fish species, dwell on the external upper side of mushroom corals (Bos, 2012; Hoeksema and Farenzena, 2012; Hoeksema and Ten Hove, 2014; Bos and Hoeksema, 2015, 2017; Montano et al., 2015). Hence, it is expected that mushroom corals may potentially offer different habitats to associated symbiotic copepods. In addition, mushroom corals species have a variety of shapes, maximum sizes, and life-history traits (Hoeksema 1989, 1991; Gittenberger et al., 2011), creating the potential for host-shape specificity of their symbionts: about 20% of the species of Fungiidae remain attached and do not become free-living (Hoeksema, 2009; Benzoni et al., 2012); different species show different maximum sizes depending on whether they have a single (monostomatous) or multiple mouths (polystomatous) (Hoeksema, 1991; Gittenberger, et al., 2011), with consequences for the space that is available for associated symbiotic organisms (Hoeksema et al., 2012; Hoeksema

2014). Therefore, mushroom coral diversity makes them suitable for a study of the host specificity of the associated copepod fauna. In this research we addressed: which Red Sea fungiid species hosted symbiotic copepods, whether the symbiotic copepods were generalists or host specific, whether different symbiotic copepods with similar or closely related hosts were evolutionarily related, addressing what could be identified as "the copepod perspective" in the relationship, and whether closely related host corals shared a similar symbiotic copepod fauna, addressing "the host perspective".

Most of the 27 species of symbiotic copepods reported from fungiid corals have been described as ectosymbionts from New Caledonia (Humes, 1973, 1996, 1997; Kim, 2003) and the Moluccas in Indonesia (Humes, 1978, 1979, 1997; Humes and Dojiri, 1983; Kim, 2007); only two species of fungiid-associated copepods were found in Madagascar (Humes and Dojiri, 1983; Kim, 2010), and none from the Red Sea until now. According to these earlier reports, *Pleuractis seychellensis* (Hoeksema, 1993) has the richest copepod fauna consisting of six species followed by *Ctenactis echinata* (Pallas, 1766) and *Sandalolitha robusta* (Quelch, 1886) with five symbiotic copepod species. Twenty-six copepod species associated with Fungiidae were restricted to this scleractinian family and were never reported from other hosts. Only one copepod species, *Asteropontius latioriger*, was found in Madagascar living on Fungiidae and Acroporidae. Twenty-one copepod species are known from only a single coral species; four copepod species (*Anchimolgus notatus* Humes, 1978, *A. punctilis* Humes, 1978, *Paramarda aculeata* Humes, 1978, and *Schedomolgus tener* (Humes, 1973)) were recorded from two host species; two copepods (*Anchimolgus pandus* Humes, 1978 and *A. latens* Humes, 1978) were found on four host species. A rather strong species-specific relationship is thus expected and we aim at

providing reliable inference on the ecological and evolutionary drivers of such host-specific symbiotic relationship using a quantitative framework to address the relationship.

2. Material and methods

2.1. The rationale of the study

The rationale of the study involved: (1) sampling a large selection of muschroom corals species in different reefs in two areas of the Red Sea, (2) extracting all the copepods living as symbionts with the corals, (3) screening already known and potentially new species from morphology, (4) using DNA-taxonomy approaches to support the morphological identification of copepods and to further delimit previously unknown species, and (5) performing ecological and evolutionary analyses on the drivers of the occurrence and of genetic diversity of the copepods in association with the corals.

2.2. Sample collection

A total of 26 coral colonies representing 13 species of fungiid corals belonging to eight genera were collected at depths ranging from 3 to 34 m at 10 different reefs located in the Central and the Southern parts (vicinity of Thuwal and Farasan Islands correspondingly) of the Saudi Arabian Red Sea (Supporting Fig. S1, Supporting Table S1). Coral colonies were collected by hand while SCUBA diving (by BWH and VNI). The corals were photographed underwater, placed in plastic bags and brought to the surface. A small amount of 70% ethanol solution in sea water was added to each bag, in order to make a 10% solution; the corals were left in the dark in this solution for half an hour to cause expulsion of the copepods from the polyps and to weaken

their attachment to the coral surface; then the corals were vigorously and thoroughly washed in the solution by agitation, to extract the symbiotic copepods (Humes, 1979; Ivanenko et al., 2008). The obtained residue was filtered through a fine net (mesh size 60 μm) and sorted out by a pipette under a dissecting microscope (Olympus SZX 7). All copepods found in the residue were preserved in 95% ethanol; after a preliminary screening to identify putative morphotypes, up to five copepod individuals representing different morphotypes and sexes from each coral host were isolated individually in 2-ml tubes for the following analyses using morphological and molecular methods. Skeletons of the host corals (Figure 1, Supporting Table S1) were cleaned from soft tissue in a solution of bleach, washed, dried, labelled, photographed and deposited in the collection of the King Abdullah University of Science and Technology (KAUST). The corals were identified based on morphological characters described and illustrated by Hoeksema (1989, 1993) and by Hoeksema and Dai (1991) and their nomenclature is following recent taxonomic revisions presented by Gittenberger et al. (2011), Benzoni et al. (2012), and Hoeksema (2014).

2.3. Morphological examination

For confocal microscopy, the exuvia (exoskeletons) of copepods remaining after extraction of DNA (see later) were kept in 2-ml vials in 96% ethanol with a small drop of glycerol, transferred to distilled water and stained with Fuchsin. The staining procedure and mounting method were adapted from Michels and Büntzow (2010) by substituting the Congo Red solution with a solution of Fuchsin (Ivanenko et al., 2012). The copepods were inspected at Lomonosov Moscow State University on an inverted Nikon A1 CLSM (Nikon Corporation, Tokyo, Japan), using a 40× oil immersion objective and lasers with wavelengths 532 and 640 nm. The laser power was set to 60%. The amplitude offset and detector gain were manually adjusted. CLSM image stacks were obtained throughout the whole animal, and the scanning software was

adjusted to perform the optimal number of scans. Image size was set for 2000×2000 dpi and the reconstruction of the external anatomy was obtained by maximum projection. The final images were adjusted for contrast and brightness using the software Adobe Photoshop CS4 (Adobe Systems, San Jose, CA, USA).

For light microscopy, the exuvia of copepods were stained with chlorazol black and studied using the "hanging drop method" with a compound microscope (Olympus CX 41) (Ivanenko and Defaye, 2004). Temporary slides were mounted using regular glass slides and cover slips were attached with small balls of plasticine. The exuvia were placed on a cover slip in a small drop of lactic acid under a dissecting microscope (Olympus SZX 7). Then the cover slip with the exuvia and small balls of plasticine attached to the corners of the glass was turned over and mounted on a glass slide, so that the exuvia did not touch the glass slide. The exuvia can be re-arranged under the dissecting microscope after removing and turning over the slide. For long-term preservation, the samples were transferred on slides in glycerol.

2.4. DNA data

We have used a non-destructive DNA extraction protocol based on Porco et al. (2010). Fixed individual copepods were transferred to wells of 0.2 mL PCR strips in 50 μL of ethanol using pipette with wide tips. Copepods were briefly centrifuged at 4000 rpm and most ethanol was removed using a pipette with 200 μL tips. An aliquot of 50 μL of lysis solution (30 mM Tris-HCl, 20 mM EDTA, 1% SDS, 0.1 mg/mL proteinase K) was added to each copepod. After two hours of incubation at 37°C, 40 μL of lysis solution was slowly transferred to new PCR strips using 10 μL pipette with thin tips to avoid picking up copepods. A standard silica-based DNA extraction kit (Diatom DNAprep 100, Isogene, Moscow, Russia) was used to extract DNA from

lysis buffer according to manufacturer's protocol for fresh blood samples. An aliquot of 100 μL of 1:1 ethanol-glycerol mix was added to copepod vouchers to preserve the morphological features.

Two molecular markers were amplified and sequenced: the mitochondrial cytochrome c oxidase subunit I (COI) and the nuclear internal transcribed spacer 2 (ITS2). We designed copepod-specific primers to improve the amplification success rate and avoid amplification of host DNA, which is often a problem when barcoding symbiotic and parasitic organisms. Amplification of the Folmer fragment of the COI gene was performed using copepod-specific forward primer LCO1490cop3 (TCITGIAAYCAYAAAGAYATYGGIAC) and universal reverse primer jgH2198 (Geller et al., 2013). PCR program was as follows: preheat at 94°C for 2 min, continued by 38 cycles of 94°C for 20 s, annealing at 45°C for 20 s, 72°C for 1 min, followed by a final elongation at 72°C for 5 min. ITS2 was amplified using pair of copepod-specific primers 58d-cop (CAGTGGATCAYTTGGCTCGGGGG) and 28r1-cop (CATTCGCCATTACTAAGGGRATCAC) using following program: preheat at 94°C for 2 min, continued by 38 cycles of 94°C for 20 s, annealing at 50°C for 20 s, 72°C for 1 min, followed by a final elongation at 72°C for 5 min. QIAGEN Multiplex PCR Kit was used. PCR products were purified with ExoSAP (Thermo Fisher Scientific) according to the manufacturer's protocol and were sequenced by Sanger technology from both ends in the KAUST Bioscience Core Lab using an ABI 3130XL Genetic Analyzer. Sequence reads were filtered and assembled using Geneious 8.1 (Kearse et al., 2012).

2.5. Phylogeny reconstruction

Sequences were obtained for 184 individuals and were aligned using the Q-ins-i algorithm implemented in MAFFT (Katoh et al., 2010) for ITS2 and by using the default settings for COI. The ITS2 alignment consisted of 134 sequences and 423 base pairs (bp); the COI alignment consisted of 167 sequences and 677 bp. COI sequences were translated into amino acids and checked for potential mistakes in the reading frame and for stop codons before using them for further analyses. No such problems were found in the COI sequences.

Alignments were reduced to unique sequences by collapsing all identical sequences into one single sequence. Sequences of different lengths were collapsed into one unique sequence if their overlapping parts were identical. In those cases, we retained the longest sequences for the analyses. Almost all sequences were obtained for the complete fragments (i.e. >350 bp for ITS2 and > 620 bp for COI). Only three sequences in the ITS2 dataset and nine sequences in the COI dataset were shorter: the shortest sequences included in the analyses were 339 bp for ITS2 and 433 bp for COI. We run our analyses on a COI dataset consisting of 120 haplotypes, an ITS2 dataset consisting of 40 haplotypes, and a concatenated COI + ITS dataset, which included the 92 terminals which had a unique combination of haplotypes from both genes (Supporting Table S2).

Maximum likelihood (ML) and Bayesian (BA) analyses were performed on individual markers as well as the combined dataset. A general time-reversible model of sequence evolution with corrections for a discrete gamma distribution (GTR + Γ) was specified for each analysis using four gamma categories. This model was selected as the best fit for all three datasets (COI, ITS, COI+ITS2) using ModelGenerator v0.85 (Keane et al., 2006).

Maximum likelihood analyses were computed with RAxML version 7.2.8 (Stamatakis, 2006). Nodal support was estimated via 1000 replicates of a non-parametric bootstrap

(Felsenstein, 1985). Bayesian analyses were performed using BEAST v.1.8.2 (Drummond et al., 2012). BEAUTi v 1.8.2 was used to generate all the xml files for the BEAST runs. An uncorrelated lognormal relaxed clock was selected for each analysis. Tree priors were selected under Coalescent Process with constant population size. Analyses were run with independent MCMC chains, which were set for 100 million generations. Sampling was set every 10,000 generations. Convergence of the reads were confirmed with Tracer v.1.5 (Rambaut and Drummond, 2007) checking that the Effective Sample Sizes (ESS) for all parameters had values higher than 200. The consensus tree based on maximum clade credibility (MCC) was obtained in TreeAnnotator v.1.6.1 with a burnin of 20% (Drummond et al., 2012).

2.6. DNA taxonomy

Molecular species delineation was performed comparing the results of three widely used methods in DNA taxonomy: the Automated Barcode Gap Discovery (ABGD) (Puillandre et al., 2012), the generalized mixed Yule-coalescent model (GMYC) (Fujisawa and Barraclough, 2013) and the Poison Tree Process (PTP) (Zhang et al., 2013).

ABGD was performed by uploading separately the COI and ITS2 alignments to the online platform (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) to test for the existence of a barcode gap in the genetic distances and then to identify groups of individuals united by shorter genetic distances than the gap. Such groups were considered equivalent to species (Puillandre et al., 2012). Since the method is based on genetic distances calculated in one marker, this approach was used only with the alignments of individual genes and not with the concatenated dataset. Distance matrices and barcoding gaps were calculated using the default parameters.

The GMYC model was performed using the R 3.3.3 (R Core Team, 2017) package *splits* v1.0-19 (Ezard et al., 2009) on ultrametric trees obtained from the COI, ITS2 and COI+ITS2 alignments. Ultrametric trees for the GMYC were obtained in two different ways, in order to control for potential biases: using the consensus tree from BEAST (as suggested by Tang et al., 2014), as well as transforming the RAxML consensus tree with the chronoMPL function (Britton et al., 2002) in the R package *ape* v.3.5 (Paradis et al., 2004).

The PTP model for species delineation was carried out on the PTP online server (http://species.h-its.org/). The method was applied to three ML trees (COI, ITS2 and COI + ITS2). The method searches for evidence of independently evolving entities by optimizing the differences in branching patterns between and within species (Zhang et al., 2013). The analyses were run with default parameters. The output from both the ML and BI optimization algorithms is reported.

After identifying the general consensus on the identity of species from DNA taxonomy, we calculated the uncorrected genetic distances within and between species using the R package *ape* for COI and for ITS2 to allow comparisons of our results with the genetic distances known in other copepod species.

2.7. Host relationships and geographical isolation

Our working hypothesis, based on existing literature, is that a species-specific relationship is present between symbiont copepods and the host corals. To investigate this, we first assessed whether the diversity of host coral species was simply correlated to the abundance of each copepod species with Pearson's correlation tests (Crawley, 2013). Then, we evaluated whether any evidence of host-associate specificity was present for the identified species of copepods on

the species of fungiid corals in our dataset by performing an association test. The rationale for the test is that if an association between copepod species and their hosts is present, then the observed number of host species for each copepod species should be lower than the number expected under the null model of no association. To obtain the distribution of the expected number of host corals under the null model of no association, we randomly shuffled the occurrence data of coral hosts for the occurrence data of each copepod species found in each individual mushroom coral. We then counted the number of host species for each copepod species in the random resampling and tested whether the observed numbers were significantly lower than those obtained from a random distribution of coral species. We repeated this simulation 1000 times and compared the distribution of the estimate number of coral species per copepod species under the null model of no association with the numbers observed in the field. We then repeated the analysis focusing also on host coral genera and not only on host species, in order to check for the strength of the association with the host at different taxonomic levels.

We also looked for a phylogenetic signal of the mushroom coral phylogeny on the occurrence of copepods. We used the most recent phylogeny reconstruction of Fungiidae by Gittenberger et al. (2011), and assessed whether for each copepod a host-phylogenetic signal was present, consistent with earlier results on fungiid-associated fauna (Hoeksema et al., 2012; van der Meij et al., 2015). As a metric of phylogenetic signal, we used Blomberg's K (Blomberg et al., 2003), which can be used for occurrence data. The rationale is that even if a strict species-specific association is not present, the occurrence of each copepod species will be more likely on phylogenetically closely related host species. We obtained the Fungiidae phylogeny using the function get.treepos, while Blomberg's K was calculated using the function phylosig, both implemented in the R package *phylotools* v0.1.2 (Revell, 2012).

Another test we performed for the host-symbiont relationship asked whether the differences in community composition of the symbiont copepods were correlated to the phylogenetic distances between host species. To address this hypothesis, we performed a matrix correlation test (Mantel test) between the matrix of Bray-Curtis dissimilarity in abundance-based community composition of copepods for each coral species, and the matrix of genetic distances between host species calculated as patristic distances from the mushroom coral phylogeny of Gittenberger et al. (2011). Patristic distances and Bray-Curtis dissimilarity matrixes were calculated using the functions cophenetic and vegdist implemented in the R packages *stats* v3.4.3 (R Core Team, 2017) and *vegan* v2.4-4 (Oksanen et al., 2017).

For each copepod species with at least 15 individuals found, we then assessed the proportion of the genetic variability that could be related to the potential association with the host species together with the effect of geographical distances. As a proxy of geographic isolation we used the localization of each coral colony in specific reefs, isolated by areas of deeper waters. Moreover, for the copepod species found in both the Southern and the Central part of the sampling area, we also assessed which proportion of the genetic variability could be related to differences between the two geographic areas. We used the adonis function in the R package *vegan* to estimate the proportions of explained variances. As a matrix of genetic distances for the analyses of each copepod species, we used the patristic distances obtained from the COI tree because we had more sequences and overall higher genetic diversity with COI than with ITS2. Host species and reef, nested within the two geographic areas when possible, were used as explanatory factors in the models for each species.

2.8. Data availability

DNA sequences are available in GenBank with accession numbers 374676 - 374842 and 401283 - 401412 (Supporting Table S2). DNA sequence data, alignments, phylogenetic trees, and images used throughout this study are publicly available on the Open Science Framework repository (ID: https://osf.io/352hc/).

3. Results

3.1. Copepod species

Based on the examination of the external morphologies used for copepod taxonomy, eight species were identified from the 184 individuals that were extracted from the mushroom corals: one Cyclopidae (cf. *Euryte*, with one individual only, unfortunately lost after extraction of DNA), one Rhynchomolgidae (one individual), two Asterocheridae of the genus *Asteropontius* (35 and 50 individuals) and four Anchimolgidae representing the genera *Prionomolgus* (two species with 1 and 17 individuals) and *Schedomolgus* (two species with 7 and 72 individuals) (Fig. 2).

Given the systematic position of the eight potential species, the phylogenies were rooted based on the distinction between the families and classes, removing the individual Cyclopidae for which no morphological information was available (Fig. 3, Supporting Figs. S2-S4).

The three DNA taxonomy approaches based on the three datasets (COI, ITS2 and COI+ITS2) provided consistent estimates of seven species (excluding the Cyclopidae) (Supporting Table S3). ABGD estimated seven entities for both COI (with prior intraspecific distances from 0.077 to 0.0215) and ITS2 markers (with prior intraspecific distances from 0.0028 to 0.0129). Lower values of prior intraspecific distances provided estimates higher than 7. This

method could not be applied on the concatenated dataset. PTP estimated seven entities for both COI and COI+ITS2 datasets, and nine entities for the ITS2 dataset. In the single marker cases the confidence interval of the solutions (COI: 7-33, mean 16.23; ITS2: 4-20, mean 9.59) was larger than in the combined dataset (COI+ITS2: 7-9, mean 7.14). GMYC did not provide a clear answer, and larger estimates than the other methods were obtained (Supporting Table S3).

Overall, the morphology and DNA taxonomy on different markers and different methods concurred to the presence of seven species of symbiotic copepods (excluding the only individual of Cyclopidae representing *Euryte* or a closely related genus). Genetic distances within these seven species-level groups were up to 8.1% in COI and 2.1% in ITS2, whereas distances between species were between 16.6% and 55.7% (up to 35.1% within each copepod family) for COI and between 2.1% and 40.9% (up to 18.5% within each family) in ITS2 (Supporting Table S4). We used these seven species to test hypotheses on species specificity of the host association and on the effect of geographic distances in structuring genetic diversity.

3.2. Symbiotic association

On the 13 mushroom coral species that hosted symbiotic copepods, from one to three copepod species were found (Fig. 4). Each copepod species occurred on as few as one and up to nine host species. The number of coral host species colonised by each copepod species correlated with the number of individuals of copepods found in our study (Pearson's $r=0.92$, $p=0.0029$).

Our results did not support any specificity of the association between symbiotic copepods and their host: each species of copepod was recovered from multiple species of fungiid corals. These empirical observations were supported by the association tests, which indicated that the numbers of observed hosts for each species (except for *Prionomolgus* sp. 2, with low sample

size) were not significantly lower than the ones obtained from a random association between corals and copepods (Table 1), neither at the level of coral species nor at the level of coral genus.

None of the copepod species showed a significant value for a phylogenetic signal of the host coral phylogenetic relationships (Supporting Table S5), and no correlation was found between similarity in copepod community composition and phylogenetic similarity between host species (Mantel test: r=-0.03, p=0.55) In addition, the genetic distances between individuals within each copepod species could be explained for only 5 to 30% by the association with the host coral, and even less by geographic isolation among reefs and geographical areas in the Red Sea (Table 2).

4. Discussion

Contrary to the previous assumptions and speculations on coral-copepod relationships based mostly on anecdotal evidence (Humes, 1985, 1994), the most relevant finding of our study is that the assumed host-specific association between symbiotic copepods and mushroom corals is unsupported. Most copepods were found on several host species and even on different genera. Moreover, the fact that the number of coral species for each copepod strictly correlated with copepod abundance suggests that also for rare copepods the diversity of host corals could be higher, and that their low number of host species might simply reflect a low sample size obtained for those copepod species. Given the relatively low number of individual copepods found in each mushroom coral (Supporting Table S1), an underestimation of host diversity could be expected for the copepod-coral association; notwithstanding this potential bias in favour of supporting an artefactual species-specificity, such specificity was clearly rejected by all the tests we performed.

We suggest that the previously assumed species level host-specificity of the relationship was probably due to a lack of information and to the fact that having few records for a potentially highly diverse group of symbiotic copepods and of host corals could misleadingly have provided a scenario of a species-specific association. The lack of a species-specific association radically changes the evolutionary perspective that was indicated for symbiotic copepods (Humes, 1985, 1994; Stella et al., 2011; Cheng et al., 2016). The assumed species-specific or at least genus-specific association meant that a co-evolutionary scenario could be hypothesised (Thrall et al., 2006), as for other symbiotic relationships known between invertebrates, such as endolithic snails, boring mussels,and gall crabs living inside scleractinian coral skeletons (Gittenberger and Gittenberger, 2011; Owada and Hoeksema, 2011; Van der Meij et al., 2015), or between invertebrates and other organisms (e.g. fig wasps, yucca, etc.). However, caution is needed here because host switching can interfere with co-evolution, even involving hosts of different phyla, subclasses, or orders, as shown by commensal shrimps (Brinkmann and Fransen, 2016; Horká et al., 2016; Hoeksema and Fransen, 2017) and corallivorous snails (Schiaparelli et al., 2015; Potkamp et al., 2017). Given the lack of host specificity of the copepods living on mushroom corals of the Red Sea at species and genus level, the high diversity of hosts cannot be suggested as a driver of the high species diversity of symbiotic copepods, as in the case of the high diversity of pollinating insects such as bees and fig-pollinating wasps related to the diversification of flowering plants (Danforth et al., 2006; Cruaud et al., 2012). Thus, the processes related to the relatively high diversity of symbiotic copepods should be searched among other factors, maybe on differential adaptations to different body parts. The lack of species-specific associations could be related to a general adaptation to the host defense systems and metabolites, with generalist chemosensors of copepods.

Different species of symbiotic copepods could still colonise different parts of the hosts and establish different associations with them: unfortunately, we could not clearly identify the localisation of each individual copepod on the host coral, and thus we do not know whether each symbiotic copepod could be ecologically diversified in its relationship, for example by colonising only the galls, the polyps, the intestinal cavity, or the soft tissues of the hosts (Stock, 1975; Humes, 1985; Dojiri, 1988; Kim and Yamashiro, 2007; Ivanenko at al., 2014). The question of the drivers of the diversification of symbiotic copepods remains open, but we can at least support that a species-specific or a genus-specific association should be ruled out.

The finding of generalist symbionts living in association with various coral species across a range of fungiid genera is also reported for other taxa, such as coral-excavating mussels (Kleemann and Hoeksema, 2002; Owada and Hoeksema, 2011), coral barnacles (Hoeksema et al., 2012), epitoniid snails (Gittenberger and Hoeksema, 2013), hydroids of the genus *Zanclea* (Montano et al., 2015), coral gall crabs (van der Meij and Hoeksema, 2013; Van der Meij et al., 2015; Hoeksema et al., 2018), sessile ctenophores (Alamaru et al., 2016, 2017), and cryptobenthic fishes (Bos, 2012; Hoeksema et al., 2012; Bos and Hoeksema, 2015, 2017). Interestingly, some coral-dwelling polychaetes of the genus *Spirobranchus*, which are commonly found on large ranges of hosts (Molodsova et al., 2016; Hoeksema and ten Hove, 2017; Perry et al., 2017), are scarcely recorded from fungiid corals (Hoeksema and ten Hove, 2014). For these generalist symbiont species, one interpretation may be that the various species of fungiid corals are functionally equivalent as far as the symbionts are concerned. Thus, it could be that all symbiont copepods are strictly associated to mushroom corals only, and not to other families of stony corals, notwithstanding the wide morphological and ecological variability of mushroom corals.

Strictly host-specific symbionts indeed exist in association with mushroom corals and can be found among endolithic snails of the genus *Leptoconchus* (Gittenberger and Gittenberger, 2011), several epitoniid snails (Gittenberger and Hoeksema, 2013), and various taxa associated with the mushroom coral *Heliofungia actiniformis* (Quoy and Gaimard, 1833), including the pipefish *Siokunichthys nigrolineatus* (Phillips, 1987) and the shrimp *Cuapetes kororensis* (Bruce, 1977) (Hoeksema et al., 2012; Hoeksema, 2017). In other words, host-specificity may occur for animals associated with Fungiidae, but it appears to be relatively rare at the host species and genus level but common at the host family level (Hoeksema et al., 2012, 2018).

All copepod species found in the Red Sea on mushroom corals are all putatively new for science, and increase the overall known diversity of symbiotic copepods (Supporting Table S6). The fact that they are still potentially undescribed suggests that a much richer diversity may exist. Morphologically, all of these copepods belong to unknown species, not matching any of the previously described species for their respective genera. Genetically, their identity as separate species was confirmed by various molecular taxonomic approaches, and by genetic distances within and between species that are comparable to those already known in other copepods (e.g. Blanco-Bercial et al., 2014; Cornils and Held, 2014; Fontaneto et al., 2015), further supporting their status as independent species. Two of the putative new copepod species belong to the genus *Asteropontius* (Siphonostomatoida: Asterocheridae), which also includes 36 congeneric species found in association with scleractinians, actiniarians and corallimorpharias in other parts of the world; only three of them were previously reported from mushroom corals: *A. caledoniensis*, *A. fungicola* and *A. latioriger* from New Caledonia, Madagascar and Moluccas (Kim, 2003, 2007, 2010). Two putative new copepod species belong to the genus *Schedomolgus* (Poecilostomatoida: Anchimolgidae), which also includes other 11 congeneric species found on scleractinian corals;

only two of the species of the genus were previously found on mushroom corals: *S. tener* and *S. dumbensis* from New Caledonia (Humes, 1973; Kim, 2010). Two other putative new species belong to another genus of the same family (Anchimolgidae), *Prionomolgus*, and differ from the only known species of this genus, *P. lanceolatus*, which was found in association with the coral *Pachyseris speciosa* (Dana, 1846) (Scleractinia incertae sedis) in northern Madagascar (Humes and Ho, 1968). The copepods of the genus *Paradoridicola* (Poecilostomatoida: Rhynchomolgidae) were previously found on Indo-Pacific alcyonacean corals only and were also unknown for the Red Sea (Humes, 1990). Among the 13 mushroom coral species recorded as hosts for copepods in the present study, eight of them (*Cycloseris costulata, Ctenactis* cf. *crassa, Danafungia horrida, Lithophyllon repanda, Pleuractis granulosa, Pleuractis* cf. *seychellensis, Pleuractis* cf. *taiwanensis, Podabacia crustacea*) were previously unknown to host symbiotic copepods, and they can now be added to the list of the previously known 11 species reported as hosts for copepods (Hoeksema et al., 2012).

The Red Sea copepods associated with mushroom corals belong to genera that are usually not found in association to the same coral family in other parts of the Indo-Pacific area (Humes, 1979, 1985; Cheng et al., 2016), whereas other copepod species of the same genera are found living in association with other coral families, even in the Red Sea (Humes, 1985). Thus, biogeographical patterns with multiple independent colonization of mushroom corals instead of host-specificity at the level of species, genus, or family could be hypothesized as relevant in the diversification of copepods associated with mushroom corals.

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Tables

Table 1. Number of copepod individuals and coral colonies, host coral species and genera for each of the most common copepod species. The results of the simulations reported for host species (and host genera) represent the proportion of the expected numbers that came out lower than the observed numbers from the resampling tests (1000 replicates) on all the occurrences of all 184 individual copepods on the host coral under a null model of no association: values smaller than 0.05 denote potentially significant associations. The other three species, found in less than 15 individuals, are not reported because no test would be meaningful with such low sample size.

species	copepods	corals	coral species	simulation for species	coral genera	simulation for genera
Asteropontius sp.1	50	12	7	0.145	6	0.168
Asteropontius sp.2	35	9	7	0.355	6	0.196
Prionomolgus sp.2	17	5	3	0.011	3	0.030
Schedomolgus sp.1	72	17	9	0.254	7	0.179

Table 2. Proportion of explained variance of genetic diversity in COI due to differences in host-coral species, and reef (nested within the Southern or the Central area, when possible) together with the p-values between parentheses. The other three species, found in less than 15 individuals, are not reported because no test would be meaningful with such low sample size.

species	host coral species	area	reef (nested in area)	unexplained
Asteropontius sp.1	0.301 (p=0.003)	0.072 (p=0.029)	0.099 (p=0.165)	0.528
Asteropontius sp.2	0.193 (p=0438)	-	0.010 (p=0.786)	0.797
Prionomolgus sp.2	0.051 (p=0.431)	-	0.114 (p=0.085)	0.835
Schedomolgus sp.1	0.166 (p=0.160)	0.122 (p=0.002)	0.118 (p=0.102)	0.594

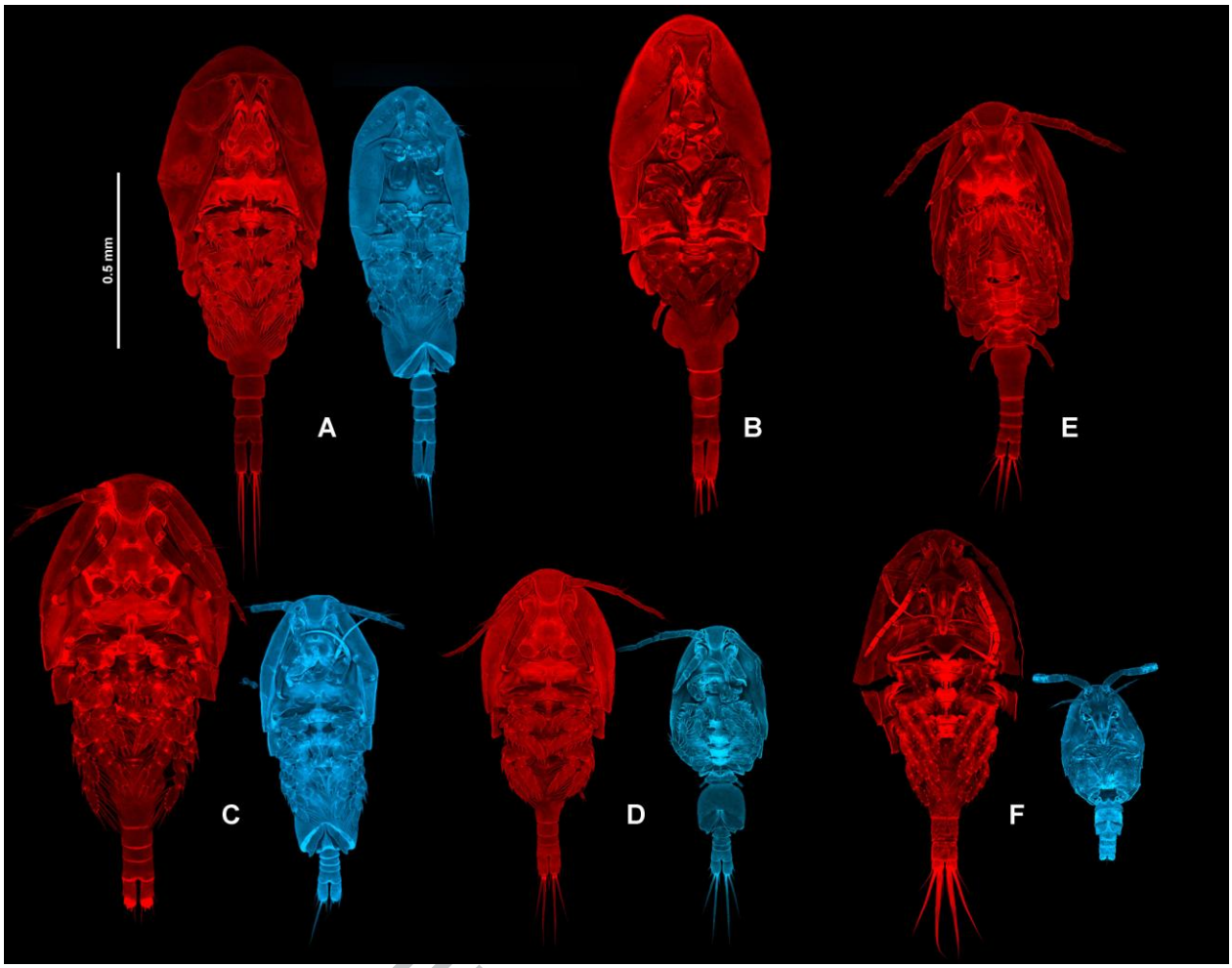
Figures

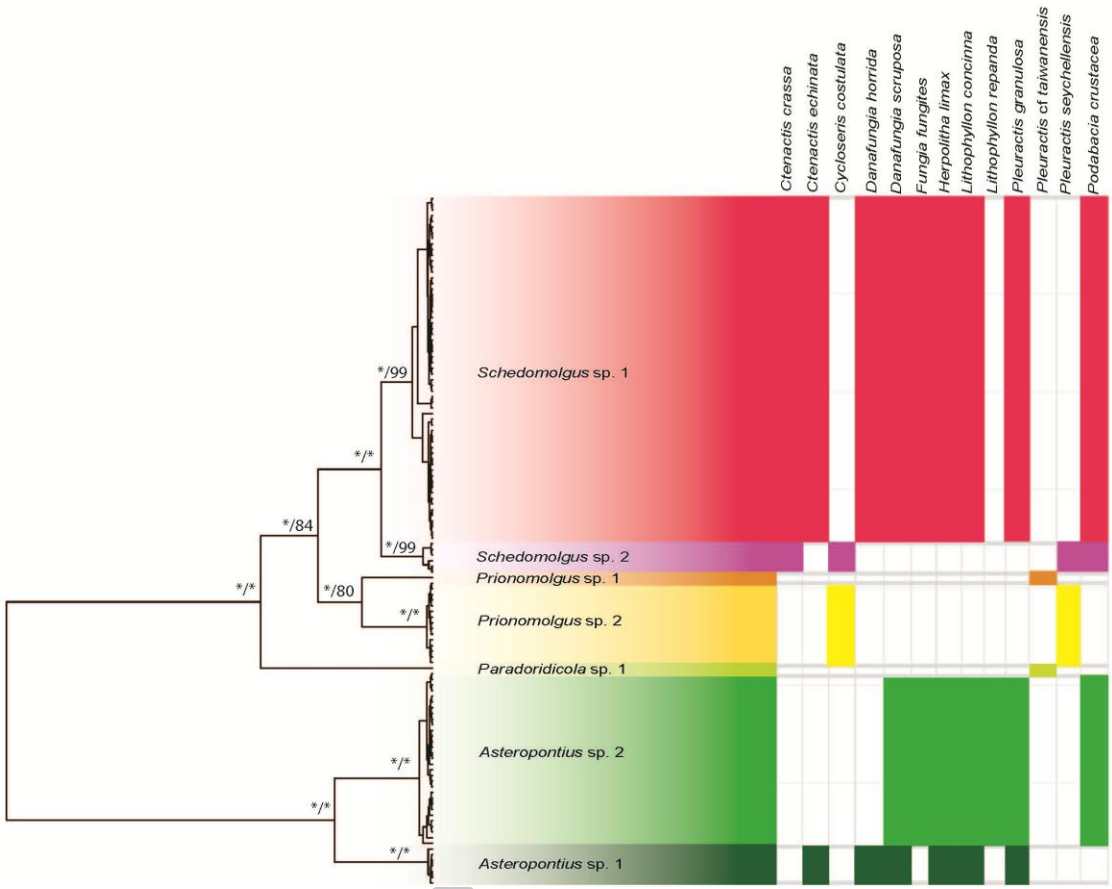
Fig. 1. Mushroom coral species recorded as host for symbiont copepods in the Red Sea. Pictures of colonies taken off Thuwal, Saudi Arabia in November 2014. A, *Ctenactis* cf. *crassa*; B, *Ctenactis echinata*; C, *Cycloseris costulata*; D, *Danafungia horrida*; E, *Danafungia scruposa*; F, *Fungia fungites*; G, *Lithophyllon concinna*; H, *Lithophyllon repanda*; I, *Pleuractis granulosa*; J, *Pleuractis* cf. *seychellensis*; K, *Pleuractis* cf. *taiwanensis*; L, *Herpolitha limax*; M, *Podabacia crustacea.*

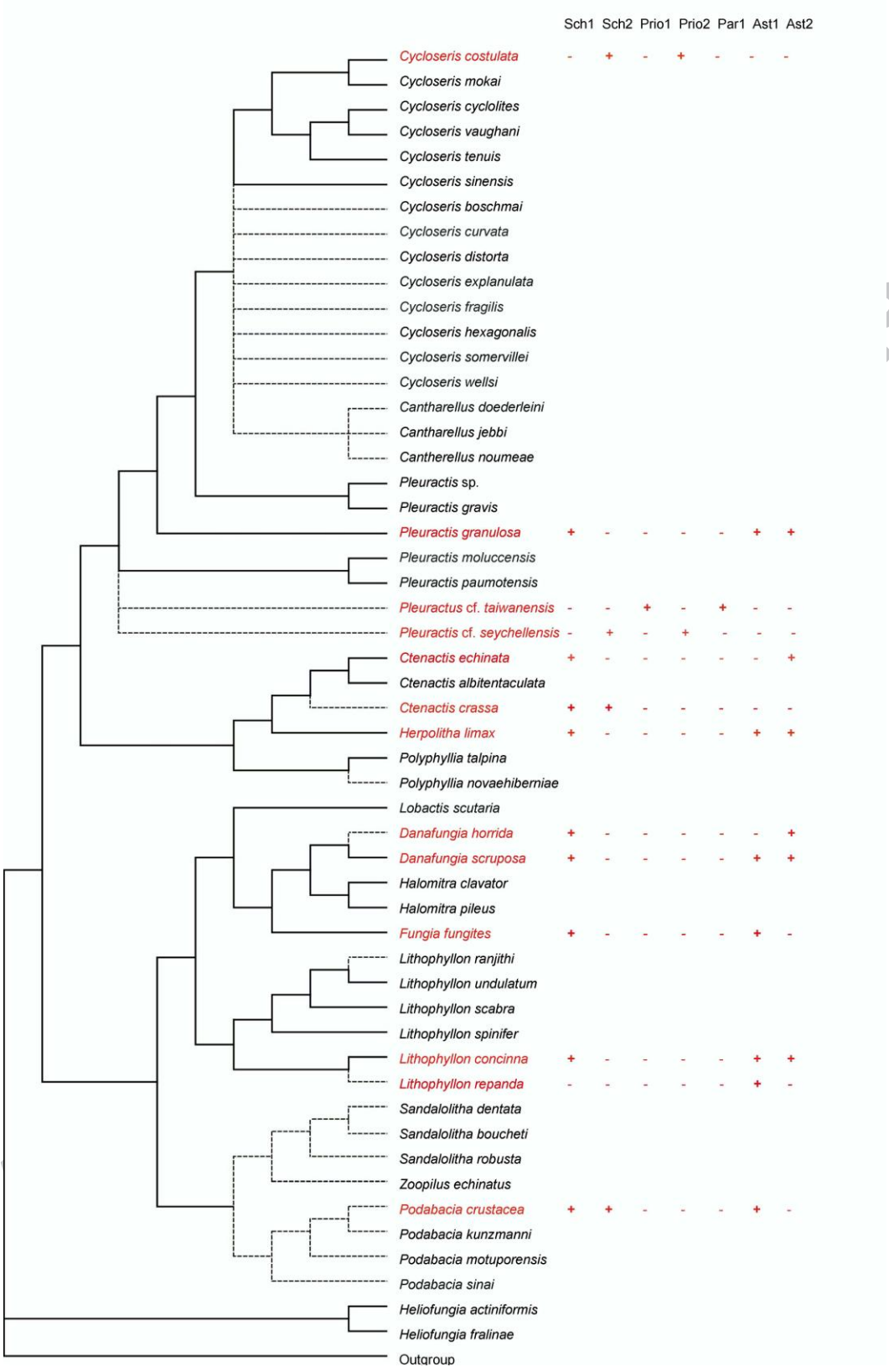
Fig. 2. Habitus of copepods found on mushroom corals of the Red Sea. Ventral view, confocal microscopy of animals after extraction of DNA (red: females, blue: males). A, *Prionomolgus* sp. 1; B, *Prionomolgus* sp. 2; C, *Schedomolgus* sp. 1; D, *Schedomolgus* sp. 2; E, *Paradoridicola* sp. 1; F, *Asteropontius* sp. 1.

Fig. 3. Phylogenetic reconstruction of the sampled copepods based on the COI analyses. The tree topology corresponds with the Bayesian analyses, whereas node values indicate support values for each clade (Bayesian posterior probabilities/ maximum likelihood bootstrap). Asterisks (*) indicate maximum support values. Entities recovered by the delineation analyses are labeled with different colors indicating their distribution across different coral host coral species. Results from other phylogenetic analyses are included as Supporting Material (Supporting Figs. 2-4).

Fig. 4. Phylogeny of mushroom coral species (after Gittenberger et al., 2011; Benzoni et al., 2012) and symbiotic copepods found on them in the Red Sea. Coral species marked in red have been recorded as host for species of Red Sea copepods: Sch1 = *Schedomolgus* sp. 1; Sch2 = *Schedomolgus* sp. 2; Prio1 = *Prionomolgus* sp. 1; Prio2 = *Prionomolgus* sp. 2; Par1 = *Paradoridicola* sp. 1; Ast1 = *Asteropontius* sp. 1; Ast2 = *Asteropontius* sp. 2; + = present, - = not observed.







Highlights

- We use DNA taxonomy on mitochondrial and nuclear markers to determine species boundaries of copepods living in the Red Sea on 13 species of mushroom corals.
- We reconstructed phylogenies of the copepods to understand their evolutionary relationships.
- The copepods show no statistically significant evidence of host-specificity or other pattern of ecological association.
- We suggest that the association between copepods and their host corals is not strict and not phylogenetically constrained.

Graphical abstract