Polyplophy of the genus Zanclea and family Zancleidae (Hydrozoa, Capitata) revealed by the integrative analysis of two bryozoan-associated species

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Abstract

The Zancleidae is a hydrozoan family that currently comprises three genera and 42 nominal species. The validity of numerous taxa in this family still needs to be assessed with integrative analyses and complete life cycle descriptions. The vast majority of its species live symbiotically with other organisms, among which cheilostomatous bryozoans are the most common hosts. These bryozoan-associated zancleids are host-specific and encompass all species of the genus Halocoryne and Zanclella, as well as several species in the genus Zanclea. Zancleids show variable morphologies, including highly reduced polyps and medusae. Their phylogenetic history is uncertain due to the often intergrading morphologies and the shortage of molecular data. In the present study, two species of Zanclea from the Indian Ocean and the Red Sea are analysed, using morphological and DNA-based approaches. Morphological analyses of the polyp and medusa stages show that, despite a general resemblance with each other and with Zancella diabolica, the two species differ in some characters, and this is supported also by molecular investigations. The DNA analyses show that the two species are monophyletic and closely related, but divergent from other Zanclea lineages. This newly recovered clade may correspond with the genus Zancella or with a cryptic genus. The lack of both morphological and molecular data for several zancleid species did not allow to address this issue. Additionally, the single- and multi-locus phylogeny reconstructions reveal that both the family Zancleidae and the genus Zanclea are polyphyletic taxa, since they are composed of at least three divergent lineages. Most zancleid species have polyps and medusae similar to other closely related taxa, and this conserved general morphology poses a challenge in the delimitation of species, genera and even families in this group. Consequently, further conjunct morphological and molecular efforts are strongly needed to clarify the diversity and evolution of the family Zancleida as a whole.

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Introduction

The Zancleidae (Russel, 1953) is a family of capitate anthoathecate hydrozoans with a complex and confusing taxonomy. Throughout the years, various authors have defined Zanclea species using conservative approaches, in some cases including multiple species into a unique ‘variable’ taxon (Russel, 1953) and in other cases describing several new species (Boero \textit{et al.}, 2000) and new genera (Boero and Hewitt, 1992). The family currently comprises 42 nominal species grouped in three genera: Zanclea Gegenbaur, 1856, Halocoryne Hadzi, 1917, and Zancella Boero & Hewitt, 1992. However, Schuchert (1996, 2010) argued against the separation of these three taxa based on morphology alone, since the characters used to discriminate them often intergrade. According to the family diagnosis given in Bouillon \textit{et al.} (2006), all species in the Zancleidae are colonial, stolonal, with creeping hydrorhiza, and with or without a perisarc sheet covering the hydrocauli and hydrorhizae. Polyps can be monomorphic or polymorphic, and in some
cases highly reduced. Medusae generally have a bell-shaped umbrella and zero, two, or four tentacles. Tentacles, when present, are armed with cnidophores, and gonads are usually interradial. Some species of *Zanclella* and *Halocoryne* have undergone reduction in the polyp and/or medusa stages (Boero et al., 2000). Petersen (1990) observed that reductions in the medusa stage are common in anthoathecate hydrozoans (e.g. in the family Corynidae) but these do not qualify as valid generic characters, since they have likely occurred independently in different species lineages after the synapomorphies defining genera evolved. For instance, all known *Halocoryne* polyps lack tentacles, but only the reproductive stage of *Halocoryne epizoica* Hadzi, 1917 is represented by a short-living eumedusoid, whereas all other species show a completely developed medusa stage (Piraino et al., 1992; Boero et al., 2000). *Zanclella* has polyps with one or two tentacles, with the exception of *Zanclella diabolica* Boero, Bouillon & Gravili 2000, which shows multiple oral and aboral tentacles, and the type species *Zanclella bryozoophila* Boero & Hewitt, 1992 reproduces through an eumedusoid, contrarily to its congeners (Boero and Hewitt, 1992; Boero et al., 2000). *Zanclella* is separated from *Zanclea* based on the reduced number of tentacles of the gastrozooids and the laterally compressed appearance of the umbrella of the medusa (Bouillon et al., 2006), but these features cannot be applied reliably to all *Zanclella* species, and, as suggested by Schuchert (1996, 2010), *Zanclella* should be regarded as a synonym of *Zanclea*. Similarly, Schuchert (2010) also highlighted that an evaluation of the relationships of *Halocoryne* species with other Zancleidae is impeded by the reduction of both the polyp and medusa stages. A proper molecular phylogeny of the three genera may therefore help resolving this uncertainty. Indeed, the taxonomic confusion is exacerbated by the fact that DNA sequences are available only for a few *Zanclea* species, whereas no molecular data have been generated so far for any *Zanclella* and *Halocoryne* species.

The majority of zancleid species live in strict association with other organisms, and the relationships with their hosts span from a presumable mutualism (Osman and Haugsness, 1981; Ristedt and Schuhmacher, 1985; Montano et al., 2017a) to parasitism (Piraino et al., 1992). Among the 30 species with a described benthic polyp stage, 16 are specifically associated with bryozoans (Boero et al., 2000; Puce et

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**Figure 1.** Maps of the investigated localities. **A:** Maldivian archipelago; **B:** Faafu Atoll; **C:** Red Sea. Numbers denote sampling sites. 1: Kika Reef; 2: Blue Cove; 3: Route 66 Reef; 4: Dhigu Reef; 5: Wall Street Reef; 6: Sunny Reef; 7: Tahla Reef; 8: Abu Gishaa Reef; 9: Abu Latt Island; 10: Sofia's Reef.
al., 2002; Pica et al., 2017), four with scleractinians (Boero et al., 2000; Pantos and Bythell, 2010; Hirose and Hirose, 2011; Montano et al., 2015a), two with octocorals (Puce et al., 2008; Varela, 2012), two with bivalves (Boero et al., 2000), two with algae (Boero et al., 2000; Galea, 2008), while four other show no substrate specificity (Agassiz, 1862; Schuchert, 2010; Peña Cantero et al., 2013). In particular, Halocoryne and Zancella species live symbiotically with bryozoans and are thought to have derived from Zanclea species associated with bryozoans and with a perisarc-free hydrorhiza protected by the skeleton of the host (Puce et al., 2002). Indeed, all species in these two genera have their naked hydrorhizae embedded in the skeleton of the host (Boero et al., 2000), and this aspect, together with the presence of highly modified polyps and medusae, and the behavioural integration with the bryozoan (Boero and Hewitt, 1992; Piraino et al., 1992), are considered as derived traits among the Zancleidae (Boero et al., 2000; Puce et al., 2002). Overall, several aspects of this enigmatic family need to be investigated further in more detail, particularly regarding the evaluation of its diversity, systematics and evolution.

The current work aims to characterise two bryozoan-associated zancleid species similar to Zancella diabolica based on specimens collected in the Indian Ocean and Red Sea. The aim is to investigate their placement within the superfamily Zancleida and present an updated phylogenetic assessment of the family Zancleidae.

Material and methods

Specimen sampling was conducted between December 2015 and May 2017 in Faafu Atoll (Maldives) and in reefs surrounding Thuwal and Al Lith (Red Sea, Saudi Arabia) (Figure 1). The presence of hydroids on bryozoans was recorded qualitatively in situ, and small fragments of bryozoans hosting hydroids were collected with hammer and chisel. After anesthetisation with menthol crystals, single hydrozoan polyps were carefully collected one by one using syringe needles, precision forceps, and micropipettes, directly from a bowl filled with seawater that was placed under a stereomicroscope. Afterwards, they were preserved in 95% ethanol for molecular analyses and 10% formalin for morphological studies. Additional portions of colonies were cultured and fed Artemia nauplii in small bowls at the MaRHE Center (Magoodhoo Island, Faafu Atoll, Maldives) in order to observe the release of medusae. Thereafter, the medusae were maintained in small bowls, and the sea water was replaced every day. The reared medusae were observed on a daily basis and some of them were fixed in 10% formalin. Morphological observations and pictures of polyps, medusae, and nematocysts were mainly performed on living specimens, whereas measurements were taken on formalin-fixed material. Photomicrographs of hydroids, medusae, and nematocysts were taken using a Leica EZ4 D stereomicroscope and a Zeiss Axioskop 40 compound microscope, both equipped with a Nikon AW 100 camera. Measurements were taken with a Nikon Eclipse E600 microscope using the NIS-Elements Viewer 4.30 software, and up to 20 specimens per species, and 50 nematocysts per type for each life stage of each species, were measured.

Tables including all available diagnostic characters for polyp and medusa stages of all the nominal zancleid species and the two species herein analysed (Supplementary tables S1, S2) were compiled by retrieving data from all the relevant literature (Agassiz, 1862; Haeckel, 1879; Browne, 1916; Hastings, 1930; Kramp, 1959; Uchida and Sugura, 1976; Calder, 1988; Hirohito, 1988; Xu et al., 1991, 2008; Boero and Hewitt, 1992; Piraino et al., 1992; Gravili et al., 1996; Migotto, 1996; Cerrano et al., 1997; Schuchert, 1996, 2010; Boero et al., 2000; Puce et al., 2002, 2008; Gershwin and Zeidler, 2003; Galea, 2008; Pantos and Bythell, 2010; Hirose and Hirose, 2011; Varela, 2012; Altuna, 2013, 2016; Peña Cantero et al., 2013; Montano et al., 2015a; Pica et al., 2017).

Total genomic DNA of ethanol-fixed samples was extracted following a protocol modified from Zietara et al. (2000). Four different molecular markers were amplified: i) a ~600 bp portion of the mitochondrial 16S ribosomal DNA gene (16S rRNA), ii) a ~700 bp portion of the mitochondrial cytochrome c oxidase subunit I gene (COXI), iii) a ~1700 bp portion of the nuclear 18S ribosomal DNA gene (18S rRNA), iv) a ~1700 bp portion of the nuclear 28S ribosomal DNA gene (28S rRNA). 16S rRNA and 28S rRNA loci were amplified using hydrozoan-specific primers following the protocols described in Cunningham and Buss (1993) and Maggioni et al. (2016). COXI and 18S rRNA genes were amplified using metazoan universal primers and the protocols proposed by Folmer et al. (1994) and Medlin et al. (1988), respectively. All PCR products were purified with Illustra ExoStar (GE Healthcare) at 37° C for
Table 1. GenBank accession numbers of the sequences included in the analyses. Newly obtained sequences are in bold.

| Family               | Genus                        | GenBank Accession Numbers |
|----------------------|------------------------------|---------------------------|
|                      |                              | 16S rRNA | COX1   | 18S rRNA | 28S rRNA |
| Asynclorynidae       | Asyncloryne ryniensis        | EU876552 | -      | EU876578 | GQ424289 |
| Cladocorynidae       | Cladocoryne haddoni          | LT593865 | LT593890 | LT593875 | LT593870 |
| Pteroclava krempfi   |                              | LT158199 | LT158209 | LT593876 | LT222039 |
| Hydrocorynidae       | Hydrocoryne miurensis        | GQ395326 | -      | -      | GQ424313 |
| Milleporidae         | Millepora alcicornis         | EU876551 | -      | AF358088 | EU879950 |
| Moerisiidae          | Moerisia inkermanica         | KT266626 | -      | KT722408 | KT757161 |
| Odessa maeotica      |                              | GQ395324 | -      | GQ424341 | GQ424314 |
| Pennariidae          | Pennaria disticha            | KF962510 | KF962169 | KF962290 | KF962370 |
| Porpitidae           | Porpita porpita              | AY935322 | GQ120060 | GQ424319 | EU883551 |
| Velessa velessa      |                              | EU305487 | KC706685 | EU876576 | EU879949 |
| Solanderiidae        | Solanderia secunda           | EU305484 | XJ121599 | EU305502 | EU305533 |
| Sphaerocorynidae     | Astrocoryne cabela           | LT714176 | -      | LT714177 | LT714178 |
| Heterocoryne caribbensis |                              | LT714185 | MH248019 | LT714186 | LT714187 |
| Sphaerocoryne sp.    |                              | LT714182 | MH248018 | LT714183 | LT714184 |
| Zancleidae           | Zanclea costata              | AY512531 | -      | EU876579 | EU879951 |
| Zanclea sango        |                              | L714107  | LT593899 | LT593874 | LT593869 |
| Zanclea sp. (DNA-XMZS) |                              | KF962532 | KF962188 | KF962298 | KF962373 |
| Zanclea prolifera    |                              | EU305488 | -      | EU272639 | EU272598 |
| Zanclea sp. 1        |                               | MH244086, | MH244068, | MH244050, | MH244014, |
|                      |                              | MH244087, | MH244069, | MH244051, | MH244015, |
|                      |                              | MH244090, | MH244072, | MH244054, | MH244018, |
|                      |                              | MH244092, | MH244074, | MH244056, | MH244110, |
|                      |                              | MH244094, | MH244076, | MH244058, | MH244112, |
|                      |                              | MH244097, | MH244079, | MH244061, | MH244115, |
|                      |                              | MH244080-085, | MH244062-067, | MH244044-049, | MH244098-103, |
|                      |                              | MH244089, | MH244071, | MH244053, | MH244107, |
|                      |                              | MH244091, | MH244073, | MH244055, | MH244109, |
|                      |                              | MH244093, | MH244075, | MH244057, | MH244111, |
|                      |                              | MH244095, | MH244077, | MH244059, | MH244113, |
|                      |                              | MH244096, | MH244078, | MH244060, | MH244114 |
| Zanclea sp. 2        |                              | MH244080-085, | MH244062-067, | MH244044-049, | MH244098-103, |
| Outgroups            | Coryne uchidai               | GQ395320 | KT981912 | GQ424332 | GQ424306 |
| Solanderiidae        | Solanderia ericopsis         | AY512520 | GU812438 | EU876556 | EU879928 |
| Milleporidae         | Millepora spp. (coral-associated) | LT714050, | LT714059, | LT714064, | LT71407, |
| Zanclea spp.         |                              | KP776810 | KP776811 | KP776811 |
| Zanclea giancarloi   |                              | FN687560-562, | FN687560-562, | FN687560-562, | FN687560-562, |
| Milleporidae         | Millepora spp.               | KF776777-779, | KT891227, | KT891230, | KT891239, |
| Solanderiidae        | Solanderia ericopsis         | AY787881 | AY787881 | AY787881 | AY787881 |
| Zanclea sessilis      |                              | AY512532 | FN687557-558, | KP776747, | KX355442 |
| Zanclea migottoi     |                              | MF538731 | MF538731 | MF538731 | MF538731 |
| Zanclea implexa      |                              | KX355448 | KX355448 | KX355448 | KX355448 |
| Zanclea sp. (DNA-977) |                              | KP776810 | KP776810 | KP776810 | KP776810 |
| Zanclea sp. (DNA-XMZS) |                              | KF962533-536 | KF962533-536 | KF962533-536 | KF962533-536 |
| Zanclea costata      |                              | EU876553, | FN687559 | FN687559 | FN687559 |
| Zanclea giancarloi   |                              | FN687560-562, | FN687560-562, | FN687560-562, | FN687560-562, |
| Milleporidae         | Millepora spp.               | KF776777-779, | KT891227, | KT891230, | KT891239, |
| Solanderiidae        | Solanderia ericopsis         | AY787881 | AY787881 | AY787881 | AY787881 |
60 min, followed by 85 °C for 15 min, and then directly sequenced in forward and reverse directions using an ABI 3730x1 DNA Analyzer (Applied Biosystems). The obtained chromatograms were visually checked and assembled using Sequencher 4.1.4 (Gene Codes). Additionally, COX1 sequences were translated using Geneious 6.1.6 (Drummond et al., 2010), in order to check for the presence of stop codons. The sequences obtained were deposited with EMBL (GenBank accession numbers: MH244044-MH244115, MH248018, MH248019), including information on specimen vouchers, collection dates, localities, and coordinates. Other available sequences belonging to representatives of the superfamily Zancleida and outgroups (Table 1) were downloaded from GenBank and added to each dataset. Sequences of each marker were aligned with MAFFT 7.110 (Katoh and Standley, 2013) using the E-INS-i option. Two datasets were generated for subsequent analyses. First, all markers were concatenated using Mesquite 3.2 (Maddison and Maddison, 2006), and appropriate partition schemes and models were determined using PartitionFinder 1.1.1 (Lanfear et al., 2012) by means of the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC). A second dataset was generated through inclusion of all 16S rRNA sequences belonging to the family Zancleidae and closely related taxa, since for most zancleid species only this marker was available in GenBank. Phylogenetic inference analyses were performed for the concatenated and 16S rRNA datasets using Bayesian inference (BI) and maximum likelihood (ML). BI analyses were performed using MrBayes 3.2.6 (Ronquist et al., 2012): four parallel Markov Chain Monte Carlo runs (MCMC) were run for 107 generations, trees were sampled every 10th generation, and burn-in was set to 25%. Maximum likelihood trees were built with RAxML 8.2.9 (Stamatakis, 2014) using 1000 bootstrap replicates. Both BI and ML analyses were run on the CIPRES server (Miller et al., 2010). Genetic distances (uncorrected p-distance, 1000 bootstrap) within and among Zancleidae species were computed for the 16S rRNA dataset using MEGA 6 (Tamura et al., 2013).

Table 2. Polyp measurements of Zanclea sp. 1 and Zanclea sp. 2. X: absent. Measurements (in μm) are rounded to the nearest unit and are shown as mean values ± standard deviations (range).

|                     | Zanclea sp. 1                                      | Zanclea sp. 2                                      |
|---------------------|----------------------------------------------------|----------------------------------------------------|
| Hydrorhiza          | Projecting out in roughly spherical clusters, with euryteles and rarely stenoteles | Projecting out for some of its length, with euryteles and rarely stenoteles |
| Polyp length        | 1227 ± 242 (803-1498)                              | 2135 ± 478 (1442-2930)                            |
| Polyp diameter (proximal) | 75 ± 14 (55-80)                               | 125 ± 20 (108-156)                               |
| Polyp diameter (distal) | 113 ± 19 (92-142)                              | 158 ± 13 (106-147)                               |
| N° of oral tentacles | 4-5                                                | 4-5                                                |
| N° of aboral tentacles | 16-21                                           | 23-30                                             |
| Oral capitula diameter | 84 ± 8 (74-93)                                 | 104 ± 6 (97-117)                                 |
| Aboral capitula diameter | 48 ± 4 (41-56)                                 | 86 ± 9 (73-104)                                  |
| Proximal capitula diameter | 36 ± 2 (32-40)                                  | 57 ± 5 (52-65)                                   |
| Medusa buds         | 1-5                                                | 1-6                                                |
| Large stenoteles length | 16 ± 1 (15-18)                                 | 20 ± 1 (18-22)                                   |
| Large stenoteles width | 13 ± 1 (12-14)                                  | 16 ± 2 (12-17)                                   |
| Medium-sized stenoteles length | X                              | 17 ± 1 (15-18)                                   |
| Medium-sized stenoteles width | X                              | 14 ± 1 (13-15)                                   |
| Small stenoteles length | 6 ± 0 (6-7)                                    | 7 ± 0 (6-8)                                      |
| Small stenoteles width | 5 ± 1 (4-6)                                    | 5 ± 0 (4-6)                                      |
| Euryteles length    | 28 ± 1 (24-29)                                   | 20 ± 1 (19-21)                                   |
| Euryteles width     | 15 ± 1 (11-16)                                   | 12 ± 1 (11-14)                                   |
| Euryteles shaft length | 92 ± 5 (84-95)                                  | 138 ± 5 (134-143)                                |
Morphology

Morphological analyses revealed that the collected samples belong to two _Zanclea_ species (here called _Zanclea_ sp. 1 and _Zanclea_ sp. 2) showing several affinities with _Zanclella diabolica_. Both species were found in the Maldives, whereas _Zanclea_ sp. 2 was also found in the Red Sea. These species live in association with cheilostome bryozoans and are similar to each other, but morphological and morphometrical analyses revealed differences in the polyp, medusa, and cnidome (Tables 2, 3; Figure 2), as shown in the ‘Systematics’ section in the Appendix. Specifically, both _Zanclea_ sp. 1 and _Zanclea_ sp. 2 have a perisarc-free hydrorhiza growing under the bryozoan skeleton, projecting out in clusters or for some of its length, and containing macrobasic holotrichous euryteles and stenoteles. Polyps are colonial, monomorphic, and with oral and aboral capitate tentacles with stenoteles of two or three size classes. Medusa buds are borne in clusters on short blastostyles arising directly form the hydrorhiza, and develop in minute medusae. Newly released medusae are characterised by a globular umbrella with several macrobasic holotrichous mastigophores, a manubrium with small oral arms, no exumbrellar nematocyst pouches, two radial canals, and two bulbs bearing tentacles equipped with cnidophores containing macrobasic apotrichous euryteles. The main differences between _Zanclea_ sp. 1 and _Zanclea_ sp. 2 are represented by the size and colour of gastrozooids, the number of aboral tentacles, the type of hydrorhizal projections, the size of euryteles and stenoteles found in the polyp stages, and the length of the manubrium of the medusa stages. These species differ from other _Zanclea_ polyps by the combination of different morphological characters (Supplementary Table S1), including the association with specific organisms, the monomorphic state of the colony, the absence of a perisarc sheet surrounding the hydrorhiza, the position of medusa buds, and the presence, type, and distribution of macrobasic euryteles. They also differ from the highly reduced polyps of _Halocoryne_ and _Zanclella_, which show zero, one, or two tentacles, with the only exception of the _Zanclea_-like polyps of _Zanclella diabolica_. The newly released medusae of _Zanclea_ sp. 1 and _Zanclea_ sp. 2 are very similar to those of _Zanclella diabolica_ and _Zanclella gomboides_ Boero, Bouillon

Table 3. Medusa measurements of _Zanclea_ sp. 1 and _Zanclea_ sp. 2. X: absent. Measurements (in μm) are rounded to the nearest unit and are shown as mean values ± standard deviations (range).

|             | _Zanclea_ sp. 1       | _Zanclea_ sp. 2       |
|-------------|-----------------------|-----------------------|
| Height      | 154 ± 6 (152-162)     | 174 ± 6 (168-180)     |
| Width       | 180 ± 7 (172-191)     | 208 ± 6 (202-215)     |
| Manubrium length | 86 ± 6 (79-97)       | 137 ± 8 (126-146)     |
| Manubrium width  | 43 ± 9 (33-52)       | 50 ± 5 (47-57)        |
| Bulbs length   | 85 ± 9 (72-99)       | 77 ± 13 (56-88)       |
| Oral arms length  | 6 ±1 (4-7)            | 5 ± 1 (4-6)           |
| Tentacles length  | 694 ± 118 (561-935)  | 510 ± 33 (480-560)    |
| N° of cnidophores | 44 ± 15 (30-70)      | 31 ± 6 (17-38)        |
| Cnidophores length  | 10-13               | 11-17                 |
| Cnidophores width      | 19 ± 1 (17-21)      | 20 ± 2 (15-25)        |
| Mastigophores length  | 13 ± 2 (10-17)      | 17 ± 1 (14-20)        |
| Mastigophores width      | 8 ± 1 (7-9)         | 8 ± 1 (7-10)          |
| Mastigophores shaft length | 35 ± 3 (30-37)    | 36 ± 1 (36-37)        |
| Euryteles length       | 6 ± 1 (5-7)        | 7 ± 1 (6-8)           |
| Euryteles width        | 5 ± 0 (4-6)        | 5 ± 0 (4-5)           |
| Euryteles shaft length  | 34 ± 4 (33-39)    | 38 ± 1 (37-40)        |
Figure 2. Polyps, medusae, and nematocysts of *Zanclea* sp. 1 (A-H) and *Zanclea* sp. 2 (I-Q). *Zanclea* sp. 1. **A**: Fertile colony; **B, C**: Undischarged and discharged macrobasic holotrichous eurytele of the polyp; **D, E**: Stenoteles of two size classes of the polyp; **F**: Newly released medusa; **G**: Macrobasic apotrichous eurytele of the medusa; **H**: Macrobasic holotrichous mastigophore of the medusa. *Zanclea* sp. 2. **I**: Fertile colony; **J, K**: Undischarged and discharged macrobasic holotrichous eurytele of the polyp; **L-N**: Stenoteles of three size classes of the polyp; **O**: Newly released medusa; **P**: Macrobasic apotrichous eurytele of the medusa; **Q**: Macrobasic holotrichous mastigophore of the medusa. Scale bar: **A, I**: ~250 μm; **F, O**: ~100 μm; **C, K**: ~30 μm; **B, D, E, G, H, L-N, P, Q**: ~15 μm.
& Gravili 2000, and highly differ from those known for all other zancleid species (Supplementary Table S2). Comparisons were nevertheless not possible with 12 zancleid species for which only the mature medusa is known (Supplementary Table S2). Therefore it was impossible to identify these specimens to species level and to formally describe Zanclea sp. 1 and Zanclea sp. 2.

**Phylogeny and genetic diversity**

The total genomic DNA was successfully extracted and four loci were amplified for all samples. The total alignments of the 16S rRNA, COX1, 18S rRNA, 28S rRNA, and concatenated datasets were 569, 607, 1682, 1629, and 4487 bp long, respectively. PartitionFinder found similar partition schemes and models under the AIC and the BIC. AIC: 16S (GTR+G+I), 18S (GTR+G+I), 28S (GTR+G+I), COX1_pos1 (GTR+G), COX1_pos2 (F81), COX1_pos3 (GTR+G). The general topology of the phylogenetic trees based on BI and ML analyses, and computed under both AIC and BIC models and partitions, were similar, and only the Bayesian topologies are shown in Figures 3 and 4. The phylogram of the superfamily Zancleida (Figure 3) is mostly concordant with previous works (Maggioni et al., 2016, 2017a). The superfamily Zancleida is well supported (Bayesian posterior probability - BPP = 1, Maximum likelihood bootstrap support - BS = 77), but the relationships within this group are, in some cases, still uncertain. The less resolved branch of the phylogenetic tree is that composed of the families Asyncorynidae Kramp, 1949, Milleporidae Fleming, 1828, Solanderiidae Marshall, 1892, and Zancleidae. The family Zancleidae appears to be polyphyletic and is split in three divergent lineages, highlighted by stars in Figure 3. A first clade (BPP = 1, BS =

### Table 4. Pairwise intra- and inter specific genetic distances (uncorrected p-distances in %) of Zanclea species based on the 16S rRNA.

Standard deviations are in parentheses. n.c.: not calculated.

|       | 1     | 2     | 3     | 4     | 5     | 6     | 7     | 8     | 9     | 10    | 11    | 12    |
|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 1. Zanclea sp. 1 | 0.5   |       |       |       |       |       |       |       |       |       |       |       |
|       | (0.2) |       |       |       |       |       |       |       |       |       |       |       |
| 2. Zanclea sp. 2 | 10.5  | 0.4   |       |       |       |       |       |       |       |       |       |       |
|       | (1.2) | (0.1) |       |       |       |       |       |       |       |       |       |       |
| 3. Zanclea costata | 12.4  | 12.1  | 3.7   |       |       |       |       |       |       |       |       |       |
|       | (1.3) | (1.3) | (0.7) |       |       |       |       |       |       |       |       |       |
| 4. Zanclea divergens | 12.7  | 13.6  | 13.1  | n.c.  |       |       |       |       |       |       |       |       |
|       | (1.4) | (1.4) | (1.3) |       |       |       |       |       |       |       |       |       |
| 5. Zanclea giancarloi | 11.7  | 11.7  | 7.1   | 11.2  | 1.0   |       |       |       |       |       |       |       |
|       | (1.3) | (1.2) | (1.0) | (1.2) | (0.3) |       |       |       |       |       |       |       |
| 6. Zanclea implexa | 11.2  | 12.4  | 7.0   | 11.0  | 5.0   | n.c.  |       |       |       |       |       |       |
|       | (1.3) | (1.3) | (1.0) | (1.2) | (0.8) |       |       |       |       |       |       |       |
| 7. Zanclea migottoi | 12.7  | 12.9  | 9.1   | 11.7  | 7.0   | 7.6   | n.c.  |       |       |       |       |       |
|       | (1.3) | (1.3) | (1.1) | (1.3) | (1.0) | (1.0) |       |       |       |       |       |       |
| 8. Zanclea proliferata | 13.7  | 12.4  | 13.9  | 12.7  | 12.7  | 13.2  | 13.2  | n.c.  |       |       |       |       |
|       | (1.4) | (1.3) | (1.3) | (1.4) | (1.3) | (1.3) | (1.3) |       |       |       |       |       |
| 9. Zanclea sessilis | 11.7  | 12.9  | 8.0   | 11.0  | 4.8   | 3.6   | 7.7   | 13.5  | 1.3   |       |       |       |
|       | (1.3) | (1.3) | (1.1) | (1.2) | (0.8) | (0.7) | (0.7) | (1.3) | (0.3) |       |       |       |
| 10. Zanclea sp. (DNA-XMZS) | 12.2  | 13.9  | 10.5  | 13.7  | 8.5   | 9.9   | 8.2   | 13.3  | 9.4   | 0.0   |       |       |
|       | (1.4) | (1.4) | (1.2) | (1.5) | (1.2) | (1.2) | (1.2) | (1.4) | (1.2) | (0.0) |       |       |
| 11. Zanclea sp. (DNA-977) | 10.7  | 12.4  | 7.9   | 10.0  | 5.0   | 3.4   | 8.4   | 12.8  | 3.9   | 9.3   | n.c.  |       |
|       | (1.3) | (1.3) | (1.1) | (1.2) | (0.9) | (0.7) | (0.7) | (1.3) | (0.7) | (1.2) |       |       |
| 12. Coral-associated Zanclea | 13.3  | 14.1  | 11.8  | 11.9  | 9.0   | 10.5  | 9.1   | 15.1  | 9.9   | 10.3  | 9.8   | 4.4   |
|       | (1.3) | (1.3) | (1.2) | (1.3) | (1.0) | (1.1) | (1.1) | (1.4) | (1.1) | (1.2) | (1.1) | (0.6) |
is composed of the type species *Zanclea costata* Gegenbaur, 1857, *Zanclea sp.* (DNA-XMZS) from the China Sea, and coral-associated *Zanclea* (*Zanclea gallii* Montano, Maggioni & Puce, 2015, *Zanclea sango* Hirose & Hirose, 2011, and *Zanclea spp.*). A second clade includes *Zanclea prolifera* Uchida & Sugiura, 1976, and is likely to be closely related to *Asyncoryne ryniensis* Warren, 1908. A third clade (BPP = 1, BS = 100) is represented by the two *Zanclea* species analysed in this work. They are monophyletic and together form a fully supported cluster. In the phylogenetic hypothesis, they are more closely related to *Solanderia secunda* (Inaba, 1892) (BPP = 0.92, BS = 72), that falls within a poorly supported group together with *Asyncoryne ryniensis*, *Millepora spp.*, *Zanclea prolifera*, and *Zanclea divergens* Boero, Bouillon & Gravili, 2000 (BPP = 0.55, BS = 48). Coral-associated *Zanclea* species cluster with *Zanclea sp.* (DNA-XMZS), as shown also in Figure 4, and with *Zanclea migottoi* Galea, 2008 (BPP = 0.92, BS = n. s.). The remaining *Zanclea* species included in the tree are the temperate water *Zanclea sessilis* Gosse, 1853, *Zanclea giancarloi* Boero, Bouillon & Gravili, 2000, *Zanclea implexa* Alder, 1856, and *Zanclea sp.* (DNA-977), which cluster together (BPP = 0.82, BS = n. s.), and *Zanclea costata*. Inter-specific genetic distances are high among all species (Table 4). The lowest values are found among the *Zanclea* species in the temperate water cluster (3.4-5 %), whereas in all other pairwise comparisons values are higher than 7%. Intra-group distances are generally low, with the exception of *Zanclea costata* (3.7 %) and coral-associated *Zanclea* (4.4 %), the latter being nevertheless composed of multiple species (Montano et al., 2015b, Maggioni et al., 2017).
Discussion

As anticipated by Boero et al. (2000), the family Zancleidae is constantly being enlarged as a result of the descriptions of new species and cryptic taxa (Maggioni et al., 2017b; Pica et al., 2017). Indeed, the integration of in-depth morphological and molecular assessments is helping re-evaluate the diversity of both Zancleidae and closely related families, taking their geographical distributions and host relationships into account if relevant (Montano et al., 2015a, 2015b, 2015c, 2017b; Maggioni et al., 2016, 2017a, 2017b; Takama et al., 2018). Moreover, ecological studies are widening the knowledge of these poorly known organisms (Montano et al., 2016; Dubé et al., 2017a, 2017b). However, the amount of undescribed or cryptic species is probably still high, due to the paucity of diagnostic morphological characters, the often intergrading morphologies (Schuchert, 2010), the incomplete knowledge of life cycles, and the specific associations or behaviour that zancleids often establish and that could, in some cases, make them difficult to spot (Boero and Hewitt, 1992; Boero et al., 2000). Similarly, the outcomes of the symbiotic relationships involving zancleids and the degree of integration with their hosts are still unexplored in most species, although recent studies unveiled patterns of host-specificity (Fontana et al., 2012; Montano et al., 2015b), characterised the anatomy of the interactions (Pantos and Hoegh-Guldberg, 2011), and discovered previously unsuspected relationships (Montano et al., 2015c, 2017a). Most of the studies have focused on the widespread and more common genus Zanclea, whereas the enigmatic genera Halocoryne and Zancella have been less investigated, with the latter dealt with in only two earlier works (Boero and Hewitt, 1992; Boero et al., 2000).

In the present study, we analysed two species whose morphology is remarkably similar to Zancella diabolica. This latter species has typical Zanclea gastrozooids but was originally placed in the genus Zancella by Boero et al. (2000) because of the peculiar bilateral symmetric bell of the newly released medusa. Indeed, this character is shared with another Zancella species, Zancella glomboides, which has nevertheless highly reduced polyps (Boero et al., 2000). The gastrozooids of the type species Zancella bryozoophila are reduced in the same way, but the sexual stage is completely different from those of the other two Zancella species, being reduced to eumedusoid (Boero and Hewitt, 1992). This inconsistent intra-generic morphological variability does not allow a secure diagnosis of Zanclea and therefore supports the synonymisation of the genus with Zanclea, at least until a robust phylogenetic hypothesis will clarify this issue. The general reduction of tentacles in Zancella and Halocoryne gastrozooids may be linked to their behavioural integration with the bryozoan hosts. For instance, the one-tentacled polyps of Zancella bryozoophila were observed feeding on food particles taken from the host lophophores (Boero and Hewitt, 1992). Furthermore, the atentaculate polyps of H. epizoica were demonstrated to feed on the lophophoral tentacles of the host (Piraino et al., 1992). The convergent evolution of these active and highly specialised feeding mechanisms may have promoted a parallel reduction of the tentacular structures, making both the reduction of medusae (Petersen, 1990) and polyps ambiguous characters to be used in generic diagnoses. On the other hand, the absence of such an extreme polyp reduction in the two Zanclea species analysed herein, as well as in Zancella diabolica and all other symbiotic Zanclea species, may reflect a less specialised feeding behaviour of the gastrozooids, which are likely passive predators, as shown, for instance, for Zanclea sessilis and Zanclea giancarloii (Miglietta et al., 2000).

Zanclea sp. 1 and Zanclea sp. 2 are monomorphic, but have a distinctive feature, which is the hydrorhiza projecting out of the bryozoan skeleton. This characteristic is found in a few other zancleids, namely Zancella diabolica, Zanclea divergens, and Zanclea eiilatensis Pica, Bastari & Puce, 2017. Another species, Zanclea exposita Puce, Cerrano, Boyer, Ferretti & Bavestrello, 2002, exhibits a perisarc-free hydrorhiza not surrounded by the bryozoan and running above the skeleton of the host (Puce et al., 2002). With the exception of Zanclea eiilatensis, these species share the presence of macrobasic holotrichous euryteles in the hydrorhiza, but Zanclea exposita and Zanclea divergens have euryteles also in the gastrozooids, and the latter species produces a typical Zanclea medusa. According to the 16S rRNA phylogram, Zanclea divergens, Zanclea sp. 1 and Zanclea sp. 2 seem to belong to a clade diverging from other Zanclea species, and the presence of exposed portions of the perisarc-free hydrorhiza may therefore have taxonomic relevance. The hydrorhizal clusters armed with nematocyst batteries were interpreted by Boero et al. (2000) as a potential first step towards colony polymorphism. If this is true, polymorphism may have arisen independently in divergent lineages within
the Zancleidae. For instance, _Zanclea divergens_, the polymorphic coral-associated _Zanclea_ (Montano et al., 2015b), and the facultatively polymorphic _Zanclea sessilis_ (Altuna, 2016) are highly divergent from each other.

The phylogenetic hypotheses presented in this work are concordant in recovering the family Zancleidae and the genus _Zanclea_ as polyphyletic taxa, even if in some cases the nodes are still not fully supported by both Bayesian inference and maximum likelihood analyses. Single- and multi-locus phylogenies show that _Zanclea proliferata_ is more closely related to _Asyncoryne ryniensis_ than to its nominal congener, even with low statistical support, confirming the findings of previous works (Nawrocki et al., 2010; Maggioni et al., 2017a). _Zanclea costata_, the type species of the genus _Zanclea_, clusters with coral-associated _Zanclea_ and _Zanclea_ sp. (DNA-XMZS) from the China Sea in the multi-locus phylogeny, suggesting that these species belong to the ‘real’ family Zancleidae and to the genus _Zanclea_.

The phylogenetic position of _Zanclea costata_ is still dubious in the single-locus tree, and the placement in _Zanclea_ of _Z. giancarloii_, _Z. sessilis_, _Z. migottoi_, _Z. implexa_, and _Zanclea_ sp. (DNA-977) needs to be confirmed with further multi-locus analyses. Similarly, the position of _Zanclea divergens_ is not fully resolved in the _16S rRNA_ phylogram, even if it seems more closely related to _Zanclea proliferata_, _Asyncoryne ryniensis_, _Millepora_ spp., _Solanderia_ spp., _Zanclea_ sp. 1 and _Zanclea_ sp. 2. The two latter species are monophyletic and closely related, as suggested by their striking morphological similarities. They form a third, divergent clade of _Zanclea_ in the concatenated analysis, with _Solanderia secunda_ as a sister taxon, demonstrating a further polyphyly of both _Zanclea_ and the Zancleidae.

According to these results, _Zanclea_ sp. 1 and _Zanclea_ sp. 2 may belong to _Zanclella_ or to a new cryptic genus, and even to a new family in the superfamily Zancleida. However, we cautiously refrain from assigning them to _Zanclella_ or from erecting new taxa, at least until genetic data of _Zanclella_ and _Halocoryne_ species will be produced and included in the analyses. Overall, the chaotic taxonomy of the Zancleidae is likely due to the general appearance of the polyps and medusae, which are often similar to those of related taxa (e.g. the gastrozooids are very similar to those of many _Solanderia_, _Millepora_ and _Corynidae_ species, while the medusae resemble those of _Asyncoryne_), and this could have contributed to the misplacement of some species and even genera.

Another relevant source of confusion is illustrated by the fact that the complete life cycle is not known for most of the 42 zancleid species. Indeed, 11 species are known only from their adult medusa stage, six from their polyp stage, and 12 from their polyp and newly released medusa stages. This is mostly due to the fact that medusa-based descriptions do not provide any information about the polyp stage and to the difficulty to find fertile colonies and rear the newly released medusa to maturity. These difficulties, along with the prior fragmentary knowledge, the rarity of some species, and the lack of DNA sequences or material suitable for molecular analyses for most taxa, pose a challenge to the resolution of the evolutionary relationships and the systematics of the Zancleidae. Therefore, we stress the need for filling the previous gaps in order to allow a further clarification of the diversity of this enigmatic family.

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**Online supplementary material**

*S1*. Polyp stage characteristics of all nominal zancleid species, *Zanclea* sp. 1, and *Zanclea* sp. 2. * Data obtained from drawings in original descriptions.

*S2*. Medusa stage characteristics of all nominal zancleid species, *Zanclea* sp. 1, and *Zanclea* sp. 2. * Data obtained from drawings in original descriptions.

*S3*. Polyp stage characteristics of all nominal zancleid species, *Zanclea* sp. 1, and *Zanclea* sp. 2. * Data obtained from drawings in original descriptions.

*S4*. Medusa stage characteristics of all nominal zancleid species, *Zanclea* sp. 1, and *Zanclea* sp. 2. * Data obtained from drawings in original descriptions.
Appendix

Systematics

Class Hydrozoa Owen, 1843
Order Anthothecata Cornelius, 1992
Suborder Capitata Kühn, 1913
Family Zancleidae Russel, 1953
Genus Zanclea Gegenbaur, 1856

Zanclea sp. 1

Material examined. Polyp stage. MA0216061: Wall Street Reef, Faafu Atoll, Maldives (3.12061°N, 73.09581°E), 10/02/2016, 19 m depth. MA0316014: Blue Cove, Faafu Atoll, Maldives (3.09383°N, 72.96651°E), 09/03/2016, 20 m depth. MA0316101: Route 66 Reef, Faafu Atoll, Maldives (3.07717°N, 72.97328°E), 21/03/2016, 8 m depth. MA0316171: Blue Cove, Faafu Atoll, Maldives (3.09383°N, 72.96651°E), 22/03/2016, 20 m depth. MA0106034: Wall Street Reef, Faafu Atoll, Maldives (3.12061°N, 73.09581°E), 16/10/2016, 17 m depth. MA0117058: Sunny Reef, Faafu Atoll, Maldives (3.14294°N, 73.01206°E), 04/02/2017, 20 m depth. MA0117174: Route 66 Reef, Faafu Atoll, Maldives (3.07717°N, 72.97328°E), 21/02/2017, 5 m depth. Medusa stage. MA021661B: 2-day-old medusae released from sample MA0216061.

Diagnosis. Polyps monomorphic, with 4-5 oral and 16-21 aboral capitate tentacles and a typical white band in the middle or in proximal part of the column. Hydrorhiza projecting out of the bryozoan skeleton with clusters of nematocysts. At release, medusa laterally compressed, with two tentacular bulbs bearing opposite tentacles; manubrium reaching the velar opening, mouth with oral arms.

Description of the polyp. Colony stolonal, growing in association with cheilostome bryozoans (Figures 2A, 5A). Hydrorhiza devoid of a perisarc sheet, reticular, crawling under the bryozoan skeleton, often projecting out at the corners of zooeciae with clusters of nematocysts (Figures 2A, 5B). Polyps monomorphic. Gastrozooids tubular, up to 1.5 mm long, 55-80 μm wide proximally and 92-142 μm distally. Mouth distal and circular, surrounded by 4-5 short oral tentacles; 16-21 additional, aboral tentacles scattered irregularly-spirally over 2/3 of the polyp column, comparatively shorter than their oral counterparts, occasionally represented by sessile capitula only (Figures 2A, 5C-D). Capitula with nematocysts and with inclusions (Figure 5E), bigger in oral tentacles (74-93 μm), and decreasing in size proximally (32-56 μm) (Figures 2A, 5C-D). Living polyps transparent, with a whitish mouth, an orange hypostome, and a typical white band in the middle or proximal half of the polyp (Figures 5A, C-D), which disappears in fixed material. Medusa buds minute, originating from the protruding hydrorhiza, and grouped in clusters of 1-5 (Figures 2A, 5F). Cnidome composed of stenoteles of two size classes (Figures 2D-E, 5G) and macrobasic holotrichous euryteles (Figures 2B-C, 5H-I). Large stenoteles (14.5-17.5 x 11.5-14 μm) in oral capitula and rarely in aboral capitula, hydrorhiza, and nematocyst clusters. Small stenoteles (5.5-7.5 x 4-6.5 μm) in all capitula, and rarely in hydrorhiza. Macrobasic holotrichous euryteles with shaft coiling along the short axis (24.5-29 x 11-16.5 μm, discharged shaft: 84-95 μm) abundant in hydrorhiza and in its projections.

Description of the newly released medusa. When released, medusae motionless for several hours. After about 10 hours, tentacles become projected outside the bell cavity. Newly released medusae small, globular, 152-162 μm high and 172-191 μm wide (Figures 2F, 5J-K). Several nematocysts scattered over the exumbrella. Manubrium 79-97 μm long and 33-52 μm wide, reaching the velar opening, with a terminal circular mouth with 3-4 arms 4-7 μm long (Figures 2F, 5L). Two opposite tentacular bulbs, initially projecting inside the bell cavity, and everted after two days. Each bulb bearing a tentacle up to 935 μm long, armed with 10-15 oval, hairy cnidophores (17-21 x 10-17 μm) borne on 30-70 μm long pedicels (Figures 2F, 5M). Each cnidophore containing 2 nematocysts. Living medusae transparent, with an orange manubrium basally, white distally (Figure 5K). After one week of cultivation, medusae with slightly longer tentacles but not displaying other differences. Adult, mature medusae not observed. Cnidome composed of macrobasic holotrichous mastigophores (Figures 2H, 5N-O) and macrobasic apotrichous euryteles (Figures 2G, 5P-Q). Macrobasic holotrichous mastigophores (7-8.5 x 6-7.5 μm, discharged shaft: 30.5-36.5 μm) scattered over the exumbrella; macrobasic apotrichous euryteles (5-7 x 3.5-6 μm, discharged shaft: 33-39 μm) in cnidophores and tentacular bulbs.

Distribution. Known from Faafu Atoll, Maldives.

Taxonomic remarks. Zanclea sp. 1 is strikingly similar to Zanclella diabolica, but it nevertheless...
Figure 5. Polyp (A-I) and medusa (J-Q) of *Zanclea* sp. 1. A: General aspect of a colony; B: Clusters of nematocysts projecting out of the bryozoan skeleton (arrowheads); C, D: Gastrozooids; E: Aboral capitulum showing nematocysts and a central inclusion; F: Medusa buds arising from hydrorhiza; G: Stenoteles of two size classes; H, I: Undischarged and discharged macrobasic holotrichous euryteles, respectively; J, K: Newly released medusae; L: Manubrium ending in a terminal mouth with oral arms; M: Cnidophores; N, O: Undischarged and discharged macrobasic holotrichous mastigophores, respectively; P, Q: Undischarged and discharged macrobasic apotrichous euryteles, respectively. Scale bars: A: 1.5 mm; B-D, F, J, K: 100 μm; E, G-I, L, M: 10 μm; N-Q: 5 μm.
shows some distinguishing features (Tables 2, 3, Supplementary Tables S1, S2). In particular, the polyps of Zanclea sp. 1 have a higher number of aboral tentacles, a distinct white band on their column, nematocyst clusters containing euryteles and rarely stenoteles, in contrast with Zanclella diabolica, which contains three stenoteles according to Boero et al. (2000). Additionally, all nematocysts in the polyp stage of Zanclea sp. 1 are smaller than in Zanclella diabolica, and the mouth of the medusa is equipped with oral arms. No other zancleid medusae show this latter feature, which could be a synapomorphy shared by Zanclea sp. 1 and Zanclea sp. 2. Alternatively, the oral arms may have gone unnoticed in other zancleid species due to their extremely small size. At this stage, it is not clear whether the morphological differences between Zanclea sp. 1 and Zanclella diabolica represent intra- or inter-specific divergence, and only a thorough re-analysis of material from the type locality and its inclusion in molecular analyses will address this issue.

**Zanclea sp. 2**

*Material examined.* Polyp stage. FB002, FB015, FB018: Abu Latt Island, Al Lith, Saudi Arabia (19.94617°N, 40.15102°E), 30/04/2017, 3 m depth. FB476: Sofia’s Reef, Al Lith, Saudi Arabia (19.79325°N, 40.40007°E), 07/05/2017, 15 m depth. KA119: Tahla Reef, Thuwal, Saudi Arabia (22.27501°N, 39.04715°E), 14/12/2015, 11 m depth. KA133: Abu Gishaa Reef, Thuwal, Saudi Arabia (22.42706°N, 39.05111°E), 16/12/2015, 17 m depth. MA0216066: Dighu Reef, Faafu Atoll, Maldives (3.08025°N, 72.98269°E), 16/02/2016, 20 m depth. MA0116036: Wall Street Reef, Faafu Atoll, Maldives (3.12061°N, 73.09581°E), 16/10/2016, 19 m depth. MA0117130, MA0117144: Kika Reef, Faafu Atoll, Maldives (3.08702°N, 72.95581°E), 13/02/2017, 14 m depth. Medusa stage. MA0216066B: 2-day-old medusae released from sample MA0216066.

*Diagnosis.* Polyps monomorphic, with 4-5 oral and 23-30 aboral capitate tentacles. Hydrorhiza projecting out of the bryozoan skeleton for some of its length. At release, medusa laterally compressed, with two tentacular bulbs bearing opposite tentacles; manubrium long, protruding from the velar opening, mouth with oral arms.

*Description of the polyp.* Colony stolonal, monomorphic, living in association with cheilostomate bryozoans (Figures 21, 6A). Hydrorhiza naked, reticular, partially crawling under the bryozoan skeleton and partially projecting out for some of its length at the borders of zooeciae (Figures 21, 6B). Gastrozoooids cylindrical, up to 3 mm long, 106-147 μm wide at base and 108-156 μm wide distally, with an apical circular mouth, 4-5 oral tentacles and 23-30 aboral tentacles arranged spirally over the distal 3/4th of the polyp (Figures 21, 6C). Tentacles short and reduced to sessile capitula in proximal part, their terminal capitations with nematocysts and inclusions (Figure 6.D). Oral tentacles with larger capitula (97-117 μm) and aboral tentacles with comparatively smaller capitula (52-104 μm), decreasing in size towards base of polyp (Figures 21, 6C). Living polyps transparent (Figures 6A, C). Medusa buds minute, borne on short blastostyles arising directly from the projected hydorhiza and grouped in clusters of up to 6 buds (Figures 21, 6E). Cnidome composed of stenoteles of three size classes (Figures 2L-N, 6F) and macrobasic holotrichous euryteles (Figures 2J-K, 6G-H). Large stenoteles (18.5-21.5 x 12-17 μm), medium-sized stenoteles (15-18 x 12.5-14.5 μm) and small stenoteles (6.75- x 4-6 μm) in capitula and rarely in hydorhiza; macrobasic holotrichous euryteles with shaft coiling along the short axis (18.5-21 x 11.5-14 μm, discharged shaft: 134-143 μm) abundant in hydorhiza.

*Description of the newly released medusa.* At release, medusae motionless for several hours and projecting tentacles outside the bell cavity after about 10 hours. Newly released medusae small, globular, 168-180 μm high and 202-215 μm wide (Figures 2O, 6I-J). Exumbrella with several scattered nematocysts. Manubrium 126-146 μm long and 47-57 μm wide, protruding from the bell cavity, with a terminal circular mouth with 4-5 arms 4-6 μm long (Figures 2O, 6K). Two opposite bulbs everted from the bell cavity after two days and bearing two tentacles up to 560 μm long, armed with 11-17 oval and hairy cnidophores (15-25 x 14-20 μm) borne on 17-38 μm long pedicels (Figures 2O, 6L). Each cnidophore with 1-3 nematocysts. Living medusae transparent, with manubrium orange proximally, and whitish for most of its length (Figure 6J). After one week of cultivation, medusae with slightly longer tentacles and no other differences. Adult, mature medusae not observed. Cnidome composed of macrobasic holotrichous mastigophores (Figures 2Q, 6M-N) and macrobasic apotrichous euryteles (Figures 2P, 6O-P). Macrobasic holotrichous mastigophores (7-10 x 6-8.5 μm, discharged shaft: 35.5-37 μm) scattered over the exumbrella; macrobasic apotrichous euryteles...
Figure 6. Polyp (A-H) and medusa (I-P) of *Zanclea* sp. 2. A: General aspect of a colony; B. Hydrorhiza partially extruding at the surface of the bryozoan host (arrowheads); C. Gastrozooids; D. Apical portion of a gastrozooid, showing four oral tentacles with nemato-cysts and central inclusions; E: Medusa buds arising from the exposed hydrorhiza; F: Large and small stenoteles; G, H: Undischarged and discharged macrobasic holotrichous euryteles, respectively; I, J: Newly released medusae; K: Terminal part of the manubrium showing a mouth with five arms; L: Cnidophores; M, N: Undischarged and discharged macrobasic holotrichous mastigophores, respectively; O, P: Undischarged and discharged macrobasic apotrichous euryteles, respectively. Scale bars: A: 1.5 mm; B, C: 200 μm; D, E, I, J: 100 μm; F, G, H, K, L: 10 μm; M-P: 5 μm.
regular and approximately spherical shape, whereas in *Zanclea* sp. 2 the hydrorhiza projects out of the bryozoan skeleton in a more irregular way and for some of its length. Moreover, the polyps in *Zanclea* sp. 2 are longer and have generally more tentacles than the other two species. Other differences are found in the size of the polyp nematocysts, as *Zanclea* sp. 2 has stenoteles of three size classes (instead of two) and smaller euryteles with a shaft length in-between the other two species. Also, the medusa of *Zanclea* sp. 2 has a longer manubrium extending further out of the velar aperture.

The morphological differences between *Zanclea* sp. 1 and *Zanclea* sp. 2 are well supported by phylogenetic and genetic distance analyses, since these two species form two fully supported monophyletic clusters.

**Distribution.** Known from Faafu Atoll (Maldives), Thuwal and Al Lith (Red Sea, Saudi Arabia).

**Taxonomic remarks.** *Zanclea* sp. 2 and *Zanclea* sp. 1 are similar to *Zanclella diabolica* in both the polyp and medusa stages. These species have *Zanclea*-like polyps, hydrorhizae projecting out of the bryozoan skeleton, minute medusa buds borne on small pedicels given off from the hydrorhiza, newly released medusae small, globular, and a similar cnidome. However, some diagnostic differences are found in both the polyp and medusa stages (Tables 2, 3, Supplementary Tables S1, S2). The hydrorhizae in *Zanclella diabolica* and *Zanclea* sp. 1 show nematocyst clusters with a more (6-8 x 4-5.5 μm, discharged shaft: 37.5-39.5 μm) in cnidophores and tentacular bulbs.