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New insights into the diversity, ecology, and evolution of the Zancleida (Hydrozoa, Cnidaria)

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ABSTRACT

The Zancleida is a poorly studied yet heterogeneous superfamily of hydrozoans that shows a wide range of morphological and ecological features. Many species in this group have complex and confusing taxonomic histories, due to the paucity of informative morphological characters, the scant available data on their life cycles, and the few molecular studies. Additionally, several species have evolved a symbiotic lifestyle and live in more or less specialised associations with a variety of other organisms, including scleractinian corals, octocorals, sponges, bryozoans, algae, and molluscs. With this work, the three symbiotic families Zancleidae, Cladocorynidae, and Sphaerocorynidae were comprehensively sampled and analysed with both morphology-based and DNA-based techniques, in order to characterise their diversity, distribution, ecology and evolution. This integrative approach allowed to shed light on the phylogenetic relationships within each family, to detect many new and cryptic species and genera, to clarify the hydrozoan-host relationships, and to better understand the evolution of peculiar morphological traits and ecological preferences. Specifically, species delimitation techniques revealed that coral-associated *Zanclea* and octocoral-associated *Pteroclava* are composed of several cryptic species with different host preference and specificity and different distribution. Moreover, ancestral state reconstructions revealed that the ancestor of coral-associated *Zanclea* was likely to be polymorphic, equipped with a perisarc-covered hydrorhiza, and host-specific. The integrative taxonomy approach also allowed to re-evaluate the phylogenetic position of some taxa, such as *Zanclea timida*, which is here moved from the family Zancleidae to the Cladocorynidae and accommodated to the newly erected genus *Pseudozanclea*, based on both morphological and molecular data. Similarly, the two new sphaerocorynid genera *Astrocoryne* and *Sphaerocorynoides* were described and the taxonomy and systematics of the whole family was clarified. The family Zancleidae is the most speciose group within the Zancleida but phylogenetic analyses revealed that this family, as well as the genera *Zanclea* and *Halocoryne*, are polyphyletic, whereas the genus *Zanclella* was recovered monophyletic. Moreover, *Zanclea* seems to harbour a cryptic diversity in other species than those associated with corals, such as in *Zanclea divergens*. Finally, an updated and well-supported phylogenetic hypothesis for the whole Zancleida was presented and used to investigate the evolution of selected morphological and ecological characters. In many cases, independent lineages within this superfamily evolved similar structures and, generally, the analysed characters seem to have been easily lost and regained during the evolution of these organisms. Overall, the inclusion of previously unsampled species and genera, as well as new phylogenetically informative molecular markers, allowed to build more robust phylogenies than in previous studies and to clarify the evolutionary history of the three analysed families. The results indicate that these often overlooked organisms harbour a great, previously unknown diversity and highlight the importance of their characterisation in changing ecosystems such as coral reefs.

RIASSUNTO

Gli Zancleida sono una superfamiglia di idrozoi poco studiata ma eterogenea, e presentano una grande varietà di caratteristiche morfologiche ed ecologiche. In questo gruppo di organismi, molte specie hanno storie tassonomiche complesse e confuse, a causa della scarsità di caratteri morfologici informativi, dei pochi dati disponibili sui cicli vitali e dei pochi studi molecolari svolti. Inoltre, molte specie hanno evoluto uno stile di vita simbiotico e vivono in associazioni più o meno strette con sclerattinie, ottocoralli, spugne, briozoi, alghe e molluschi. Con questo lavoro, le tre famiglie simbiotiche Zancleidae, Cladocorynidae e Sphaerocorynidae sono state ampiamente campionate ed analizzate utilizzando tecniche morfologiche e molecolari, al fine di descrivere la loro diversità, distribuzione, ecologia ed evoluzione. Questo approccio integrativo ha permesso di chiarire le relazioni filogenetiche all'interno di ciascuna famiglia, di individuare specie e generi nuovi e criptici, di analizzare le relazioni tra idrozoo e ospite, e di meglio comprendere l'evoluzione di tratti morfologici e preferenze ecologiche peculiari. In particolare, le tecniche di delimitazione di specie hanno rivelato che *Zanclea* associata a coralli e *Pteroclava* associata ad ottocoralli sono composte da numerose specie criptiche con diversa preferenza e specificità di ospite e diversa distribuzione. Inoltre, le ricostruzioni degli stati ancestrali di alcuni caratteri hanno mostrato che l'antenato comune alle specie di *Zanclea* associate a coralli era probabilmente polimorfico, con l'idroriza ricoperta da perisarco e ospite-specifico. L'approccio di tassonomia integrativa ha anche permesso di rivalutare la posizione filogenetica di alcuni taxa, come *Zanclea timida*, la quale è stata spostata dalla famiglia Zancleidae alla famiglia Cladocorynidae e posizionata nel nuovo genere *Pseudozanclea*. In maniera simile, sono stati descritti due nuovi generi di Sphaerocorynidae, ovvero *Astrocoryne* e *Sphaerocorynoides*, e la tassonomia e sistematica di tutta la famiglia è stata rivisitata e chiarificata. La famiglia Zancleidae è il gruppo più ricco in specie all'interno degli Zancleida, ma le analisi filogenetiche hanno mostrato che questa famiglia, così come due dei generi che la compongono (*Zanclea* e *Halocoryne*) sono polifiletici, mentre il genere *Zanclella* è stato dimostrato essere monofiletico. Inoltre, *Zanclea* sembra ospitare una diversità criptica in altre specie oltre quelle associate a coralli, come in *Zanclea divergens*. Infine, è stata presentata un'ipotesi filogenetica aggiornata e ben supportata per l'intera superfamiglia Zancleida e questa ipotesi è stata utilizzata per studiare l'evoluzione di alcuni caratteri morfologici ed ecologici. In molti casi, linee evolutive indipendenti hanno sviluppato strutture simili e, in generale, i caratteri analizzati sembrano essere facilmente persi e riacquisiti durante l'evoluzione. Complessivamente, l'inclusione nelle analisi di specie e generi prima non analizzati e di nuovi marcatori molecolari ha permesso di costruire ipotesi filogenetiche meglio supportate rispetto a studi precedenti e di fare chiarezza sulla storia evolutiva delle tre famiglie analizzate. I risultati ottenuti indicano che questi organismi spesso trascurati ospitano una grande diversità, precedentemente non nota, e sottolineano l'importanza del loro studio e caratterizzazione, in particolare in ecosistemi soggetti a grandi cambiamenti, quali le scogliere coralline.

CHAPTER 1

General Introduction

1.1. PHYLOGENETIC OVERVIEW OF THE ZANCLEIDA

The phylum Cnidaria is a diverse group of organisms comprising three major clades: Anthozoa, Endocnidozoa and Medusozoa (Collins 2009). Relationships among these clades have been debated for decades, but, only recently, increased taxon sampling and the use of phylogenomic approaches (Zapata et al. 2015, Kayal et al. 2017) allowed the clarification of the evolutionary history of the Cnidaria. Specifically, the three major clades were confirmed to be monophyletic, with the Anthozoa placed as sister group of other cnidarians and the Endocnidozoa as sister group of the Medusozoa. Also the relationships within the Medusozoa have been subject of several studies in the last years (Collins 2002, Marques and Collins 2004, Collins et al. 2006) and the most recent hypotheses support the presence of two main lineages, one composed of the Staurozoa, Cubozoa and Scyphozoa and another one including Hydrozoa (Zapata et al. 2015, Kayal et al. 2017). The higher-level systematics of Hydrozoa is still a subject of debate and authors subdivided them in several different ways and at different levels. However, the use of molecular tools and the study of life history and anatomical features allowed a clarification of these relationships and Hydrozoa are now known to be composed of two main clades: the Hydroidolina and the Trachylina (Collins 2000, Collins 2002, Marques and Collins 2004, Collins et al. 2006, Cartwright et al. 2008, Collins et al. 2008, Kayal et al. 2013, Kayal et al. 2015, Kayal et al. 2017). The Hydroidolina counts three orders, two of which were demonstrated to be monophyletic (Leptothecata and Siphonophora) (Dunn et al. 2005, Cartwright et al. 2008, Leclère et al. 2009, Maronna et al. 2016). The third order, the Anthoathecata, was constantly recovered as polyphyletic (Cartwright et al. 2008, Kayal et al. 2015) and is composed of the Aplanulata, i.e. species lacking a ciliated planula, four groups of Filifera (Filifera I-IV), i.e. species whose tentacles have nematocysts organised more or less uniformly (filiform tentacles), and the Capitata, i.e. species with tentacles equipped with a distal cluster of nematocyst (capitate tentacles). Previously, all species with capitate tentacles were united within the suborder Capitata, but molecular analyses revealed that aplanulate hydrozoans belong to an independent evolutionary lineage and the term ‘Capitata’ now refers to non-aplanulate species with capitate tentacles (Collins et al. 2005, Nawrocki et al. 2010, Nawrocki et al. 2013). The Capitata has been studied in a number of classic morphological works in which cladistics analyses were also used to reconstruct the phylogeny of the group (e.g. Petersen (1990), Rees (1957)), but only the use of molecular tools allowed to clarify, at least partially, the evolution of capitate hydrozoans (Collins et al. 2005, Nawrocki et al. 2010). Specifically, Nawrocki et al. (2010) recognised two superfamilies, namely the Corynida, including the families Corynidae Johnston 1836 and Cladonematidae Gegenbaur 1857, and the Zancleida, composed of the remaining capitate families, with the exception of some taxa that are still classified as Capitata *incertae sedis* (Nawrocki et al. 2010, Schuchert 2010). In their phylogenetic assessments, Collins et al. (2005) and Nawrocki et al. (2010) were mostly interested in the relationships among the different capitata families, and both focused their attention mainly on the systematics of the family Corynidae. The Zancleida families were therefore poorly sampled at genus and species level and, in addition, the statistical support was not high for many relationships, likely due to molecular marker resolution and undersampling of taxa. Consequently, the evolutionary relationships within the Zancleida were

not completely elucidated, especially at species level. However, the available phylogenies are concordant in recovering all families monophyletic, with the exception of the Zancleidae that were found polyphyletic due to the divergent position of *Zanclea prolifera* Uchida & Sugiura 1976 (Nawrocki et al. 2010, Fontana et al. 2012) and may be characterised by a further non-monophyly at both family and genus level (Fontana et al. 2012).

1.2. DIVERSITY OF THE ZANCLEIDA

According to WoRMS (last accessed on October 30, 2017) the Zancleida comprehends at least 14 families and roughly 115 valid species, of which more than the 30% belong to the family Zancleidae Russel 1953. However, this is likely to be an underestimation of the real diversity of the group, due to the absence of clear synapomorphies, the ineffectiveness of morphological characters for the identification of closely related species, and the lack of molecular studies for most of the taxa (Nawrocki et al. 2010, Schuchert 2010, Miglietta et al. 2015). Indeed, DNA sequences are available for less than 20 species of Zancleida and for many species a single molecular marker was sequenced, generally the hydrozoan barcode gene *16S rRNA* (https://www.ncbi.nlm.nih.gov/taxonomy, last accessed on October 30, 2017), leaving most of the Zancleida genetic diversity still unexplored. The most diverse group in terms of species is the genus *Zanclea* with 34 nominal species and possibly with a greater amount of still unknown species (Boero et al. 2000). Other taxa previously considered species-rich, subsequently underwent numerous synonymisations, such as the genus *Millepora* (Boschma 1948), but only integrative approaches including molecular assessments and micro and macro-morphological studies could possibly clarify the diversity of all Zancleida groups. Moreover, little is known about the intra-specific genetic diversity of the Zancleida species and studies are limited to few taxa. For instance, previous studies focused on the invasive species *Pennaria disticha* and *Moerisia* sp. and found high levels of genetic variation, in some cases also ascribable to the presence of cryptic species (Meek et al. 2013, Miglietta et al. 2015). Several other studies investigated the genetic diversity of *Millepora* species in order to study, for instance, patterns of connectivity, intra-colony genetic diversity and phenotypic plasticity (de Souza et al. 2017, Dubé et al. 2017, Schweinsberg et al. 2017). Finally, another work investigated the genetic diversity and host-specificity of a recently characterised group of coral-associates belonging to the genus *Zanclea*, finding that these hydrozoans harbour a low species diversity and suggesting that a cosmopolitan and genus-specific association may occur between *Zanclea* and their coral hosts (Fontana et al. 2012) .

Although few studies investigated the genetic diversity of the Zancleida, the morphological diversity is well known and different species show a wide range of morphologies, with some peculiar apomorphies and convergences with other hydrozoan groups. The Zancleida, as well as the other Anthoathecata, lacks a theca during the polyp stage, and this feature has to be interpreted as plesiomorphic (Cartwright and Nawrocki 2010). However, many species have maintained a chitinous perisarc covering the hydrorhiza and pedicels, even though some symbiotic taxa have completely lost the ability to produce chitinous structures (Puce et al. 2008).

Regarding the colony organisation, most species have an encrusting growth shape, with polyps directly arising from the stolonal hydrorhiza. This is the case, for instance, for all species of the Zancleidae, Asyncorynidae Kramp 1949, Cladocorynidae Allman 1872, and Sphaerocorynidae Prévot 1959 (Bouillon et al. 2006). However, certain taxa have evolved peculiar growth strategies, such as the Milleporidae Fleming 1828 that are able to deposit a calcium carbonate skeleton and grow massive colonies, the Solanderiidae Marshall 1892 that have an arborescent growth and an internal skeleton made of anastomosed chitinous fibers, or the Hydrocorynidae Rees 1957 that have colonies issued from a chitinised hydrorhizal stolonal plate. Few species have evolved a tendency towards solitary polyps, such as the Moerisiidae Poche 1914 and Halimedusidae Arai & Brinckmann-Voss 1980, with the polyps often anchored to the substrate through pedal discs and, finally, the Porpitidae Goldfuss 1818 live the entirety of their life cycles as planktonic organisms. All Capitata species are characterised by the presence of stenoteles in their cnidome, but also other nematocysts are commonly found in this group, including desmonemes, mastigophores, and euryteles, and the cnidome is considered of great taxonomic importance in the identification of morphologically similar species (e.g. Boero et al. (2000)). Even if one of the major distinguishing feature of the Capitata is the presence of capitate tentacles, in few cases other types of tentacles can occur in the Zancleida, such as moniliform and filiform tentacles, and also highly derived tentacles such as the ramified capitate tentacles (Prévot 1959). Moreover, tentacles can be organised in oral and aboral whorls: most species have both, but in some cases aboral tentacles are not present (Petersen 1990). Some species in the families Zancleidae, Milleporidae, and Porpitidae underwent a polyp reduction and/or specialisation, leading to polymorphic colonies with highly modified polyps (Bouillon et al. 2006). Boero et al. (2000) hypothesised that a first step towards colony polymorphism in the Zancleidae could be represented by the presence of nematocyst clusters on the hydrorhiza, such as in *Zanclea divergens* Boero, Bouillon & Gravili 2000 or in *Zanclella diabolica* Boero, Bouillon & Gravili 2000. 'Real' polymorphic colonies are nevertheless composed of other specialised polyps than the feeding ones (gastrozooids), such as protective polyps without mouths and usually with reduced tentacles (dactylozooids) and sexual polyps (gonozooids) (Bouillon et al. 2006). The position where the reproductive structures are carried varies among polyps belonging to different species. Medusa buds can be borne in the oral half of the polyp as well as in the proximal half or even on blastostyles arising directly from the hydrorhiza and the medusa stage can be reduced to different levels. The majority of the Zancleida species produce free-swimming medusae or medusoids, but *Cladocoryne* Rotch 1871, *Heterocoryne* Wedler & Larson 1986 and *Solanderia* Duchassaing & Michelin 1846 species underwent a further reduction and carry fixed medusoids that are developed either on the polyp or on the coenosarc. Some authors hypothesised that some of the morphological features described above may be related to the symbiotic lifestyle undertaken by some species. For instance, according to this idea, an evolutionary trend may be detected in certain symbiotic taxa, with a tendency towards polymorphism, a perisarc-free and host-protected hydrorhiza, and stolonal medusa buds (Boero et al. 2000, Puce et al. 2002). However, the lack of well-supported and comprehensive phylogenies for these groups did not allow to test this hypothesis.

1.3. MOLECULAR TOOLS TO STUDY THE DIVERSITY OF THE ZANCLEIDA

Despite the general morphological diversity of the superfamily, closely related species often exhibit either limited and intergrading anatomical diversification (e.g. Boero et al. (2000), Nawrocki et al. (2010)) or high intra-specific variation (e.g. Tepper et al. (2012)), making therefore difficult the identification based on morphology only. Moreover, it is often necessary to examine both mature polyps and medusae to take taxonomic decisions, but this could be challenging for species that are rare or difficult to cultivate. In the last ten years, the mitochondrial *16S rRNA* has been demonstrated to be an efficient molecular marker to be used in discriminating several hydrozoans at species (e.g. Collins et al. (2005), Moura et al. (2008), Schuchert (2010)) and also population level (de Souza et al. 2017). For this reason, the *16S rRNA* has been proposed as the universal DNA barcode for hydrozoans, in replacement of the typical metazoan barcode cytochrome *c* oxidase subunit I (*COX1*) (Moura et al. 2008, Zheng et al. 2014). Previous molecular phylogenetic studies including the Zancleida were based on the *16S rRNA* alone or in conjunction with other ribosomal markers, including the nuclear *18S* and *28S rRNA* (Collins et al. 2005, Nawrocki et al. 2010, Fontana et al. 2012), and the use of nuclear along with mitochondrial markers increased the nodal support also at higher taxonomic levels (Collins et al. 2006). Further studies revealed that other molecular markers are useful for resolving the evolutionary relationships of hydrozoans at different taxonomic levels. For instance, mitochondrial *COX1* and cytochrome *c* oxidase subunit III *COX3*, and nuclear internal transcribed spacer (*ITS*) are highly variable (e.g. Schuchert (2014), Peña-Cantero and Sentandreu (2017)) and may be used for species identification and population-level studies, whereas other markers, such as the Calmodulin and the Elongation Factor 1α genes evolve slowly and are more suitable for the resolution of deep nodes (Lindner et al. 2008, Miglietta et al. 2009, Miglietta and Cunningham 2012, Postaire et al. 2016). When studying the species diversity in closely related taxa, the phylogenetic trees alone may be not sufficient to obtain conclusive results and a possible solution to this problem could be the use of species delimitation techniques (Fontaneto et al. 2015). These methods must be intended as part of an 'integrative taxonomy' approach since, when possible, taxonomy should include multiple approaches based on genetics, morphology, ecology, behaviour, geography, as well as any other sources of available information (Dayrat 2005, Fontaneto et al. 2015). However, in some cases the DNA may be the only solution for cryptic species delimitation and several species delimitation tools are now available, with the most used being the Automatic Barcode Gap Discovery (Puillandre et al. 2012), the Generalised Mixed Yule Coalescent (Pons et al. 2006, Fujisawa and Barraclough 2013), and the Poisson Tree Processes (Zhang et al. 2013). It has also been proposed to treat the DNA itself in the same way as a morphological character and to include DNA sequence information in species description, along with morphological, ecological and behavioural data (Jörger and Schrödl 2013). A possible shortcome of these DNA taxonomy methods is that some level of incongruence is likely to be present between different methods and there are no obvious ways to decide which result can be trusted (Fontaneto et al. 2015). In order to prevent the establishment of parallel nomenclatures due to the use of molecular characters alone, a novel nomenclatural system has been proposed for morphospecies showing a further structured genetic diversity, with the possibility to easily

transfer a molecular operational taxonomic unit into the formal nomenclature as soon as new relevant evidences are provided (Morard et al. 2016). The methods and approaches described above, when taken together, are therefore promising tools to better characterise the diversity of taxonomically complex groups, such as the Zancleida.

1.4. ECOLOGY OF THE ZANCLEIDA

Species in the superfamily Zancleida are worldwide distributed and occur in both freshwater and marine environments. The majority of taxa live in the tropical and subtropical waters of the Atlantic, Pacific, and Indian Oceans (e.g. Millard (1975), Schuchert (2010), Hirohito (1988), Wedler and Larson (1986), Calder (1988)), whereas other are found in temperate seas (e.g. Bouillon et al. (2004)). Similarly, most species are found in shallow to moderately deep waters, but some can reach considerable depths, such as *Zanclea* sp. collected at 500 meters deep (Bouillon et al. 2000) and *Rosalinda incrustans* Kramp 1947 reaching a depth of more than 800 meters (Mastrototaro et al. 2016). In few cases, representative of the Zancleida are also found in polar seas, such as *Zanclea hicksoni* (Stepanjants 1972) living in the Southern Ocean (Peña Cantero et al. 2013). Few species are known to inhabit freshwater and brackish areas, and examples are represented by *Halmomises lacustris* von Kennel 1891 described from a freshwater lagoon, and other moerisiid species living in saline lakes and estuaries (von Kennel 1891, Jankowski 2001, Jankowski et al. 2007) . Some species have a limited distribution, as in the case of *Halocoryne epizoica* Hadzi 1917, a Mediterranean endemic (Piraino et al. 1992), or *Millepora laboreli* Amaral 2008, exclusively living in a specific region of Northern Brazil (Amaral et al. 2008). Conversely, other species have a wide distributional range and can be found both in tropical and temperate seas. For instance, the family Porpitidae has a circumglobal distribution in tropical to temperate waters (Schuchert 2010) and, similarly, *Pennaria disticha* Goldfuss 1820 can be found in the Mediterranean Sea, Red Sea, Caribbean Sea, Atlantic, Indian and Pacific Ocean (Miglietta et al. (2015), Miglietta et al. (in preparation)). This latter species has been classified as one of the most common introduced species in Hawaii (Coles et al. 2006) and molecular analyses demonstrated that it is actually a species complex and that multiple cryptic species may have been introduced independently in the Hawaii. Also another species, *Moerisia* sp. (likely *Moerisia lyonsi* Boulenger 1908), is considered introduced in some localities. This species is thought to be native to the Ponto-Caspian region but since 1993 has been recorded in the San Francisco Estuary (Mills and Sommer 1995, Mills and Rees 2000, Meek et al. 2013).

Even if the Hydrozoa are generally considered substrate generalist, many species live symbiotically with other organisms, and these associations may range from simple epibiosis to specialised mutualism and parasitism (Puce et al. 2008). Within the Zancleida, three group are known to live in more or less strict symbiotic relationships with their hosts, and are the family Sphaerocorynidae, associated with sponges, the genus *Pteroclava* Weill 1931, associated with octocorals and other hydrozoans, and the family Zancleidae, in which the majority of species live in association with bryozoans, scleractinian corals, octocorals, bivalves, and algae. In most cases, these association are poorly known from an ecological point of view and almost no information

is available about the possible outputs of the symbioses for the two organisms. With the exception of few *Zanclea* species, all other symbiotic taxa can be considered as partial endosymbionts of their hosts, since their hydrorhizae grow embedded by host tissues and skeleton (Puce et al. 2008, Pantos and Bythell 2010), and this suggests that, at least in some cases, the two organisms may be highly integrated. However, almost no studies are available on the host specificity of these associates and how the selectivity for a certain host is achieved. For instance, for other specifically associated hydrozoan species, the selectivity is known to be present at the larval stage and the larva needs specific compounds of bacterial origin in order to settle on the host and then metamorphosise (Müller and Leitz 2002). Changes in this recognition system may possibly result in ecological speciations due, for instance, to host shift and consequent reproductive isolation.

Overall, the ecology of Zancleida hydrozoans has been poorly investigated, since studies traditionally focused on leptothecate and filiferan species (Gili and Hughes 1995). However, some data are available for a number of species, regarding for instance the seasonality of *Zanclea divergens* (Di Camillo et al. 2008), the habitat preference of *Pteroclava krempfi* (Billard 1919) (Montano et al. 2016), the susceptibility of *Millepora* spp. to diseases, predation, and other disturbances (Lewis 2006), and the feeding behaviour of *Pennaria disticha* (Clark and Cook 1986). Moreover, different authors showed that different species belonging to the Zancleidae may have a beneficial effect on their bryozoan hosts. Osman and Haugsness (1981) and Ristedt and Schuhmacher (1985) showed that the associations between *Zanclea* sp. and *Celleporaria brunnea* (Hincks 1884) in Southern California and between *Zanclea* sp. and *Rhynchozoon larreyi* (Audouin 1826) in the Red Sea, respectively, benefit both the hydrozoans and the bryozoans by increasing their competitive ability and survival. Piraino et al. (1992) noted that also the fact that the bryozoan *Schizobrachiella sanguinea* (Norman 1868) was a successful competitor in the investigated area (Southern Italy) may be linked to its association with *Halocoryne epizoica*, even if the latter displays a peculiar parasitic behaviour towards the host. Finally, a recent work showed that also scleractinian corals could take advantage from their associated *Zanclea* hydrozoans, since they could gain protection against certain predators and diseases (Montano et al. 2017). Therefore, there is increasing evidence that the Zancleida symbiotic species may highly benefit their hosts, and the comprehension of these mechanisms as well as the characterisation of these associates are of fundamental importance, especially for those species living in ecosystems currently subjected to various severe stressors and changing environmental conditions, such as coral reefs (Hughes et al. 2017).

1.5. AIMS OF THE WORK

There is now wide consensus in affirming that current climate changes are reshuffling the diversity and geographic distributions of species worldwide (Parmesan & Yohe 2003), but even if these changes are well-documented for some species, they are likely to be completely unknown for the majority of living species, especially for those poorly studied at taxonomical and ecological level. Moreover, the effects of species decline and distributional changes on biotic interactions remain poorly understood, especially in symbiotic systems (Dunn et al. 2009) and the understanding of the taxonomic composition and interactions is a first step in the

clarification of the functional biology and performance thresholds of these systems (Gates and Ainsworth 2011).

The Zancleida comprehends species with known or suspected important ecological roles in both tropical and temperate areas, such as the haermatipic *Millepora* and the bryozoan-associated *Zanclea* (e.g. Osman and Haugsness (1981), Lewis (2006)), and some other symbiotic species may have important roles in influencing the performance limits of their hosts (Montano et al. 2017). However, the understanding of the diversity of this group, as well as the relationships with the hosts in symbiotic species are still highly incomplete. Therefore, the aim of this work is to widen the current knowledge of the taxonomy, systematics and evolution of the superfamily Zancleida, using an integrative approach and focusing on the three families Zancleidae, Cladocorynidae, and Sphaerocorynidae, which are known to be mainly composed of symbiotic species. Specifically, this study is addressed at:

1. The characterisation of the diversity, host-specificity and evolution of coral-associated *Zanclea* species.
2. The elucidation of the phylogeny of the Cladocorynidae with an emphasis on the octocoral-associated *Pteroclava krempfi* species complex.
3. The clarification of the taxonomy and evolutionary relationships within the sponge-associated family Sphaerocorynidae.
4. The reconstruction of a well-supported phylogenetic hypothesis for the superfamily Zancleida and, in particular, for the family Zancleidae, in conjunction with the study of the evolution of important ecological and morphological characters.

1.6. REFERENCES

Amaral FM, Steiner AQ, Broadhurst MK, Cairns SD (2008) An overview of the shallow-water calcified hydroids from Brazil (Hydrozoa: Cnidaria), including the description of a new species. *Zootaxa* 1930: 56-68.

Boero F, Bouillon J, Gravili C (2000) A survey of *Zanclea*, *Halocoryne* and *Zanclella* (Cnidaria, Hydrozoa, Anthomedusae, Zancleidae) with description of new species. *Italian Journal of Zoology* 67: 93-124.

Boschma H (1948) The species problem in *Millepora*. *Zoologische Verhandelingen* 1: 1-115.

Bouillon J, Gravili C, Gili J-M, Boero F (2006) *An Introduction to Hydrozoa*. Publications Scientifiques du Museum, Paris.

Bouillon J, Medel MD, Pagès F, Gili JM, Boero F, Gravili C (2004) Fauna of the Mediterranean Hydrozoa. *Scientia Marina* 68: 5-438.

Bouillon J, Pagès F, Gili J-M, Palanques A, Puig P, Heussner S (2000) Deep-water Hydromedusae from the Lacaze-Duthiers submarine canyon (Banyuls, northwestern Mediterranean) and description of two new genera, *Guillea* and *Parateclaia*. *Scientia Marina* 64: 87-95.

Calder DR (1988) Shallow-Water Hydroids of Bermuda: The Athecatae. *Royal Ontario Museum Life Sciences Contributions* 148: 1-107.

Cartwright P, Evans NM, Dunn CW, Marques AC, Miglietta MP, Schuchert P, Collins AG (2008) Phylogenetics of Hydroidolina (Hydrozoa: Cnidaria). *Journal of the Marine Biological Association of the United Kingdom* 88: 1663-1672.

Cartwright P, Nawrocki AM (2010) Character evolution in Hydrozoa (phylum Cnidaria). *Integrative and Comparative Biology* 50: 456-472.

Clark SD, Cook CB (1986) Inhibition of nematocyst discharge during feeding in the colonial hydroid *Halocordyle distich*a (= *Pennaria tiarella*): the role of previous prey-killing. *The Biological Bulletin* 171: 405-416.

Coles SL, Kandel FLM, Reath P, Longenecker K, Eldredge LG (2006) Rapid assessment of nonindigenous marine species on coral reefs in the main Hawaiian Islands. *Pacific Science* 60: 483-507.

Collins AG (2000) Towards understanding the phylogenetic history of Hydrozoa: Hypothesis testing with 18S gene sequence data. *Scientia Marina* 64: 5-22.

Collins AG (2002) Phylogeny of Medusozoa and the evolution of cnidarian life cycles. *Journal of Evolutionary Biology* 15: 418-432.

Collins AG (2009) Recent insights into cnidarian phylogeny. *Smithsonian Contributions to the Marine Sciences* 139-149.

Collins AG, Bentlage B, Lindner A, Lindsay D, Haddock SHD, Jarms G et al., Cartwright P (2008) Phylogenetics of Trachylina (Cnidaria: Hydrozoa) with new insights on the evolution of some problematical taxa. *Journal of the Marine Biological Association of the United Kingdom* 88: 1673-1685.

Collins AG, Schuchert P, Marques AC, Jankowski T, Medina M, Schierwater B (2006) Medusozoan phylogeny and character evolution clarified by new large and small subunit rDNA data and an assessment of the utility of phylogenetic mixture models. *Systematic Biology* 55: 97-115.

Collins AG, Winkelmann S, Hadrys H, Schierwater B (2005) Phylogeny of Capitata and Corynidae (Cnidaria, Hydrozoa) in light of mitochondrial 16S rDNA data. *Zoologica Scripta* 34: 91-99.

Dayrat B (2005) Towards integrative taxonomy. *Biological Journal of the Linnean Society* 85: 407-415.

de Souza JN, Nunes FL, Zilberberg C, Sanchez JA, Migotto AE, Hoeksema BW et al., Lindner A (2017) Contrasting patterns of connectivity among endemic and widespread fire coral species (*Millepora* spp.) in the tropical Southwestern Atlantic. *Coral Reefs*: 1-16.

Di Camillo C, Bavestrello G, Valisano L, Puce S (2008) Spatial and temporal distribution in a tropical hydroid assemblage. *Journal of the Marine Biological Association of the United Kingdom* 88: 1589-1599.

Dubé CE, Boissin E, Maynard JA, Planes S (2017) Fire coral clones demonstrate phenotypic plasticity among reef habitats. *Molecular Ecology* 26: 3860-3869.

Dunn CW, Pugh PR, Haddock SH (2005) Molecular phylogenetics of the siphonophora (Cnidaria), with implications for the evolution of functional specialization. *Systematic Biology* 54: 916-935.

Dunn RR, Harris NC, Colwell RK, Koh LP, Sodhi NS (2009) The sixth mass coextinction: are most endangered species parasites and mutualists? *Proceedings of the Royal Society of London B: Biological Sciences* 276: 3037-3045.

Fontana S, Keshavmurthy S, Hsieh HJ, Denis V, Kuo C-Y, Hsu C-M et al., Chen CA (2012) Molecular evidence shows low species diversity of coral-associated hydroids in *Acropora* corals. *PloS One* 7: e50130.

Fontaneto D, Flot J-F, Tang CQ (2015) Guidelines for DNA taxonomy, with a focus on the meiofauna. *Marine Biodiversity* 45: 433-451.

Fujisawa T, Barraclough TG (2013) Delimiting species using single-locus data and the Generalized Mixed Yule Coalescent approach: a revised method and evaluation on simulated data sets. *Systematic Biology* 62: 707-724.

Gates RD, Ainsworth TD (2011) The nature and taxonomic composition of coral symbiomes as drivers of performance limits in scleractinian corals. *Journal of Experimental Marine Biology and Ecology* 408: 94-101.

Gili J-M, Hughes R (1995) The ecology of marine benthic hydroids. *Oceanography and Marine Biology* 33: 351-426.

Hirohito ES (1988) *The Hydroids of Sagami Bay*. Publications of the Biolological Laboratory, Imperial Household, Tokyo.

Hughes TP, Barnes ML, Bellwood DR, Cinner JE, Cumming GS, Jackson JB et al., Morrison TH (2017) Coral reefs in the Anthropocene. *Nature* 546: 82-90.

Jankowski T (2001) The freshwater medusae of the world-a taxonomic and systematic literature study with some remarks on other inland water jellyfish. *Hydrobiologia* 462: 91-113.

Jankowski T, Collins AG, Campbell R (2007) Global diversity of inland water cnidarians. *Hydrobiologia* 595: 35-40.

Jörger KM, Schrödl M (2013) How to describe a cryptic species? Practical challenges of molecular taxonomy. *Frontiers in Zoology* 10: 59.

Kayal E, Bastian B, Pankey MS, Ohdera A, Medina M, Plachetzki DC et al., Ryan JF (2017) Comprehensive phylogenomic analyses resolve cnidarian relationships and the origins of key organismal traits. *PeerJ Preprints*.

Kayal E, Bentlage B, Cartwright P, Yanagihara AA, Lindsay DJ, Hopcroft RR, Collins AG (2015) Phylogenetic analysis of higher-level relationships within Hydroidolina (Cnidaria: Hydrozoa) using mitochondrial genome data and insight into their mitochondrial transcription. *PeerJ* 3: e1403.

Kayal E, Roure B, Philippe H, Collins AG, Lavrov DV (2013) Cnidarian phylogenetic relationships as revealed by mitogenomics. *BMC Evolutionary Biology* 13: 5.

Leclère L, Schuchert P, Cruaud C, Couloux A, Manuel M (2009) Molecular phylogenetics of Thecata (Hydrozoa, Cnidaria) reveals long-term maintenance of life history traits despite high frequency of recent character changes. *Systematic Biology* 58: 509-526.

Lewis JB (2006) Biology and ecology of the hydrocoral *Millepora* on coral reefs. *Advances in Marine Biology* 50: 1-55.

Lin M, Xu Z, Huang J, Guo D, Wang C (2016) Taxonomic notes on Hydroidomedusae (Cnidaria) from South China Sea I: Family Eucodoniidae (Anthomedusae). *Zoological Systematics* 41: 48-53.

Lindner A, Cairns SD, Cunningham CW (2008) From offshore to onshore: multiple origins of shallow-water corals from deep-sea ancestors. *PloS One* 3: e2429.

Maronna MM, Miranda TP, Cantero ÁLP, Barbeitos MS, Marques AC (2016) Towards a phylogenetic classification of Leptothecata (Cnidaria, Hydrozoa). *Scientific Reports* 6: 18075.

Marques AC, Collins AG (2004) Cladistic analysis of Medusozoa and cnidarian evolution. *Invertebrate Biology* 123: 23-42.

Mastrototaro F, Aguilar R, Chimienti G, Gravili C, Boero F (2016) The rediscovery of *Rosalinda incrustans* (Cnidaria: Hydrozoa) in the Mediterranean Sea. *Italian Journal of Zoology* 83: 244-247.

Meek MH, Wintzer AP, Shepherd N, May B (2013) Genetic diversity and reproductive mode in two non-native hydromedusae, *Maeotias marginata* and *Moerisia* sp., in the upper San Francisco Estuary, California. *Biological Invasions* 15: 199-212.

Miglietta MP, Cunningham CW (2012) Evolution of life cycle, colony morphology, and host specificity in the family Hydractiniidae (Hydrozoa, Cnidaria). *Evolution* 66: 3876-3901.

Miglietta MP, Odegard D, Faure B, Faucci A (2015) Barcoding Techniques Help Tracking the Evolutionary History of the Introduced Species *Pennaria disticha* (Hydrozoa, Cnidaria). *PloS One* 10: e0144762.

Miglietta MP, Schuchert P, Cunningham CW (2009) Reconciling genealogical and morphological species in a worldwide study of the Family Hydractiniidae (Cnidaria, Hydrozoa). *Zoologica Scripta* 38: 403-430.

Millard NAH (1975) Monograph on the Hydroida of Southern Africa. *Annales of the South African Museum* 68: 1-513.

Mills C, Rees JT (2000) New observations and corrections concerning the trio of invasive hydromedusae *Maeotias marginata* (= *M. inexpectata*), *Blackfordia virginica*, and *Moerisia* sp. in the San Francisco Estuary. *Scientia Marina* 64: 151-155.

Mills C, Sommer F (1995) Invertebrate introductions in marine habitats: two species of hydromedusae (Cnidaria) native to the Black Sea, Maeotias inexspectata and Blackfordia virginica, invade San Francisco Bay. *Marine Biology* 122: 279-288.

Montano S, Allevi V, Seveso D, Maggioni D, Galli P (2016) Habitat preferences of the *Pteroclava krempfi*-alcyonaceans symbiosis: inner vs outer coral reefs. *Symbiosis* 72: 225-231.

Montano S, Fattorini S, Parravicini V, Berumen ML, Galli P, Maggioni D, Arrigoni R, Seveso D, Strona G. (2017). Corals hosting symbiotic hydrozoans are less susceptible to predation and disease. *Proceedings of the Royal Society B: Biological Sciences* 284: 20172405.

Morard R, Escarguel G, Weiner AK, André A, Douady CJ, Wade CM et al., Quillévéré F (2016) Nomenclature for the nameless: a proposal for an integrative molecular taxonomy of cryptic diversity exemplified by planktonic foraminifera. *Systematic Biology* 65: 925-940.

Moura CJ, Harris DJ, Cunha MR, Rogers AD (2008) DNA barcoding reveals cryptic diversity in marine hydroids (Cnidaria, Hydrozoa) from coastal and deep-sea environments. *Zoologica Scripta* 37: 93-108.

Müller WA, Leitz T (2002) Metamorphosis in the Cnidaria. *Canadian Journal of Zoology* 80: 1755-1771.

Nawrocki AM, Collins AG, Hirano YM, Schuchert P, Cartwright P (2013) Phylogenetic placement of *Hydra* and relationships within Aplanulata (Cnidaria: Hydrozoa). *Molecular Phylogenetics and Evolution* 67: 60-71.

Nawrocki AM, Schuchert P, Cartwright P (2010) Phylogenetics and evolution of Capitata (Cnidaria: Hydrozoa), and the systematics of Corynidae. *Zoologica Scripta* 39: 290-304.

Osman RW, Haugsness JA (1981) Mutualism among sessile invertebrates: a mediator of competition and predation. *Science* 211: 846-848.

Pantos O, Bythell JC (2010) A novel reef coral symbiosis. *Coral Reefs* 29: 761-770.

Peña-Cantero ÁL, Sentandreu V (2017) Phylogenetic relationships of endemic Antarctic species of *Staurotheca* Allman, 1888 (Cnidaria, Hydrozoa). *Polar Biology* 40: 301-312.

Peña Cantero ÁL, Boero F, Piraino S (2013) Shallow-water benthic hydroids from Tethys Bay (Terra Nova Bay, Ross Sea, Antarctica). *Polar Biology* 36: 731-753.

Petersen KW (1990) Evolution and taxonomy in capitate hydroids and medusae (Cnidaria: Hydrozoa). *Zoological Journal of the Linnean Society* 100: 101-231.

Piraino S, Bouillon J, Boero F (1992) *Halocoryne epizoica* (Cnidaria, Hydrozoa), a hydroid that "bites". *Sci. Mar.* 56: 141-147.

Pons J, Barraclough TG, Gomez-Zurita J, Cardoso A, Duran DP, Hazell S et al., Vogler AP (2006) Sequence-based species delimitation for the DNA taxonomy of undescribed insects. *Systematic Biology* 55: 595-609.

Postaire B, Magalon H, Bourmaud CAF, Gravier-Bonnet N, Bruggemann JH (2016) Phylogenetic relationships within Aglaopheniidae (Cnidaria, Hydrozoa) reveal unexpected generic diversity. *Zoologica Scripta* 45: 103-114.

Prévot E (1959) Morphologie et évolution des structures tentaculaires chez les hydraires gymnoblastes Capitata. *Recueil des Travaux de la Station Marine d'Endoume* 17: 91-126

Puce S, Cerrano C, Boyer M, Ferretti C, Bavestrello G (2002) *Zanclea* (Cnidaria: Hydrozoa) species from Bunaken Marine Park (Sulawesi Sea, Indonesia). *Journal of the Marine Biological Association of the United Kingdom* 82: 943-954.

Puce S, Cerrano C, Di Camillo CG, Bavestrello G (2008) Hydroidomedusae (Cnidaria: Hydrozoa) symbiotic radiation. *Journal of the Marine Biological Association of the United Kingdom* 88: 1715-1721.

Puillandre N, Lambert A, Brouillet S, Achaz G (2012) ABGD, Automatic Barcode Gap Discovery for primary species delimitation. *Molecular Ecology* 21: 1864-1877.

Rees WJ (1957) Evolutionary trends in the classification of capitate hydroids and medusae. *Bulletin of the British Museum of Natural History, Zoology* 4: 455-534.

Ristedt H, Schuhmacher H (1985) The bryozoan *Rhynchozoon larreyi* (Audouin, 1826)-A successful competitor in coral reef communities of the Red Sea. *Marine Ecology* 6: 167-179.

Schuchert (1996) *The marine fauna of New Zealand: Athecate hydroids and their medusae (CNidaria: Hydrozoa)*. New Zealand Oceanographic Institute.

Schuchert P (2010) The European athecate hydroids and their medusae (Hydrozoa, Cnidaria): Capitata part 2. *Revue Suisse de Zoologie* 117: 337-555.

Schuchert P (2014) High genetic diversity in the hydroid *Plumularia setacea*: a multitude of cryptic species or extensive population subdivision? *Molecular Phylogenetics and Evolution* 76: 1-9.

Schweinsberg M, Tollrian R, Lampert KP (2017) Inter-and intra-colonial genotypic diversity in hermatypic hydrozoans of the family Milleporidae. *Marine Ecology* 38: e12388.

Tepper C, Squiers L, Hay C, Gorbach D, Friend D, Black B et al., Strychar K (2012) Cryptic species: a mismatch between genetics and morphology in *Millepora*. *Marine Science* 2: 57-65.

von Kennel J (1891) On a freshwater medusa. *Journal of Natural History* 8: 259-263.

Wedler E, Larson R (1986) Athecate hydroids from Puerto Rico and the virgin Islands. *Studies on Neotropical Fauna and Environment* 21: 69-101.

Zapata F, Goetz FE, Smith SA, Howison M, Siebert S, Church SH et al., France SC (2015) Phylogenomic analyses support traditional relationships within Cnidaria. *PloS One* 10: e0139068.

Zhang J, Kapli P, Pavlidis P, Stamatakis A (2013) A general species delimitation method with applications to phylogenetic placements. *Bioinformatics* 29: 2869-2876.

Zheng L, He J, Lin Y, Cao W, Zhang W (2014) 16S rRNA is a better choice than COI for DNA barcoding hydrozoans in the coastal waters of China. *Acta Oceanologica Sinica* 33: 55-76.

CHAPTER 2

The enigmatic coral-associated *Zanclea* (Cnidaria, Capitata): diversity, biogeography, relationship with the hosts, and evolution

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2.1. ABSTRACT

Scleractinian reef corals have recently been acknowledged as the most numerous host group found in association with hydrozoans belonging to the genus *Zanclea*. However, knowledge of the diversity of *Zanclea* species associated with scleractinians is just beginning. To date, four nominal species are known from the Indian and Pacific Ocean, but the evolutionary history and phylogeny of these species, as well as their ecological preferences, are still far from being elucidated. With this work, *Zanclea* colonies associated with more than 30 coral genera from several localities were studied using an integrative approach. Despite morphological analyses recovered three morphospecies, phylogenetic analyses, species delimitation techniques and population genetics recovered seven well supported evolutionary lineages akin to species. Specifically, each of the three morphospecies is a cryptic species complex and a recently proposed 'molecular nomenclature' was therefore used to name each species. The level of host specificity is variable in different species and possible causes of the observed patterns are discussed. Population genetics analyses showed that populations from the Red Sea are generally more isolated with respect of other populations and this isolation could be also responsible for a speciation event. Overall, these results boost the knowledge of the diversity, distribution, and evolution of a previously overlooked association that could play important roles in coral symbiomes.

2.2. INTRODUCTION

Most hydrozoans (Cnidaria, Hydrozoa) are considered substrate generalists, which live indiscriminately on many different types of biotic and abiotic substrates. However, several hydroids are symbiotic with metazoan organisms, such as sponges, cnidarians, molluscs, annelids, bryozoans, crustaceans, echinoderms, tunicates and vertebrates (Gili and Hughes 1995, Boero and Bouillon 2005, Puce et al. 2008a). In other cases, hydrozoans can also establish specific associations with non-metazoan organisms, including sea weeds, marine plants, and microorganisms (Puce et al. 2008a, Abouna et al. 2015, Stabili et al. 2017). These associations range from simple epibioses to strict symbioses, in which the hydroid settles on the living epithelium or inside the tissue of the host (Puce et al. 2007). Hydroids in the genus *Zanclea* Gegenbaur 1857 are perceived as highly specialised symbionts, with a worldwide distribution including the Atlantic Ocean (Calder 1988), Indo-Pacific Ocean (Kramp 1968), Southern Ocean (Stepanjants 1972), Mediterranean Sea (Gravili et al. 1996), and Red Sea (Ristedt and Schuhmacher 1985), and with a depth preference ranging from the intertidal zone (Pantos and Bythell 2010, Hirose and Hirose 2011, Montano et al. 2013) up to a depth of 500 m (Bouillon et al. 2000). Of all 34 nominal species ascribed to the genus *Zanclea*, a dozen have been described exclusively based on medusa specimens collected using plankton nets (Haeckel 1879, Uchida and Sugiura 1976, Xu et al. 1991, Gershwin and Zeidler 2003, Xu et al. 2008). The remaining *Zanclea* species, identified through observation of the polyp and medusa stages, are known to have a preference for living substrates, usually forming symbiotic relationships with marine organisms such as bivalves, octocorals and bryozoans (Gravili et al. 1996, Boero et al. 2000, Puce et al. 2002, Puce et al. 2007, Puce et al. 2008a, Puce et al. 2008b). Scleractinian reef corals are traditionally known to host many taxa of associated organisms (Stella et al. 2011, Hoeksema et al. 2012) and, recently, several studies have revealed that the genus *Zanclea* is an additional component of this plethora of symbioses (Boero et al. 2000, Pantos and Bythell 2010, Hirose and Hirose 2011, Montano et al. 2013). Even though this association was originally reported in Mozambique (Millard and Bouillon 1974, Millard 1975) and Papua New Guinea (Boero et al. 2000), the first comprehensive description of a *Zanclea*-scleractinian association was provided only in 2010 by Pantos and Bythell (2010). After this work was published, the number of studies increased, with papers focusing on different aspects of this association such as ecology, taxonomy, physical interactions, and geographical distribution (Hirose and Hirose 2011, Pantos and Hoegh-Guldberg 2011, Fontana et al. 2012, Montano et al. 2013, Montano et al. 2014, Montano et al. 2015a, Montano et al. 2015b). The association with scleractinians currently involves the four species *Zanclea gilii* Boero, Bouillon & Gravili 2000, *Zanclea margaritae* Pantos & Bythell 2010, *Zanclea sango* Hirose & Hirose 2011, *Zanclea gallii* Montano, Maggioni & Puce 2014, and some as yet unidentified species (Boero et al. 2000, Pantos and Bythell 2010, Hirose and Hirose 2011, Fontana et al. 2012, Montano et al. 2015b). All those species belong to the "polymorpha group", showing colonies of hydroids consisting of both gono-gastrozooids and retractile dactylozooids (Boero et al. 2000). The geographic distribution of this association includes the Red Sea (Montano et al. 2014) and several Indo-Pacific regions such as Australia, Indonesia, Taiwan, Japan and the Republic of Maldives (Pantos and Bythell 2010, Hirose and Hirose 2011, Fontana et al. 2012, Montano et

al. 2013). The host range currently includes 24 scleractinian genera belonging to seven families, with a total of 33 scleractinian species involved (Montano et al. 2015b). Thus, reef-building corals are the host group with the highest number of species found in association with *Zanclea* species. Fontana et al. (2012) recently proposed a genus-specific association between *Zanclea* and scleractinians. However, whereas *Z. gallii*, *Z. margaritae*, and the unidentified *Zanclea* specimens studied by Fontana et al. (2012) settle locally on the genus *Acropora* Oken 1815 (Pantos and Bythell 2010, Montano et al. 2015a), Z. *sango* is a more generalist species living on the genera *Pavona* Lamarck 1801 and *Psammocora* Dana 1846 and shows a widespread distribution (Hirose and Hirose 2011, Montano et al. 2015a). Except for these preliminary data, no other information at the species level is available regarding the host-specificity and diversity of *Zanclea* associated with scleractinians. Differences in the hydroid colony, the absence and presence of perisarc and the cnidome of both the polyp and medusa stages are the morphological features generally used to identify *Zanclea* species (Boero et al. 2000). Two of the coral-associated described species (i.e. *Z. gilii* and *Z. sango*) are equipped with eurytele capsules in their cnidome and *Z. sango* also has perisarc covering the hydrorhiza and pedicels (Boero et al. 2000; Hirose Hirose, 2011), while the two other species (i.e. *Z. margaritae* and *Z. gallii*) are free of euryteles and perisarc (Pantos and Bythell 2010, Montano et al. 2015a). The physical interaction with the host has been analysed only in the Australian species *Z. margaritae*, and, in this case, the perisarc-free colony attaches itself to the coral skeleton through desmocytes (Pantos and Hoegh-Guldberg 2011), similarly to how hydrozoans and corals are normally attached to their perisarc and skeleton, respectively (Marcum and Diehl 1978, Muscatine et al. 1997).

Knowledge regarding the molecular phylogenetic relationships among *Zanclea* species associated with scleractinians is still far from complete. In fact, with the exception of the recent description of *Z. gallii* based on an integrated morpho-molecular approach (Montano et al. 2015a), the three other *Zanclea* species were described only through the study of their morphological characters (Boero et al. 2000, Pantos and Bythell 2010, Hirose and Hirose 2011). At present, mitochondrial and nuclear phylogenetic analyses have shown that all the available sequences of *Zanclea* associated with scleractinians form a monophyletic lineage clearly separated from the type species *Zanclea costata* Gegenbaur 1857 (Fontana et al. 2012, Montano et al. 2015a). Within this cohesive group, both *Z. sango* and *Z. gallii* were recovered as distinct monophyletic lineages based on partial *16S* gene sequences, with the latter species closely related but molecularly separated from the unidentified *Acropora*-associated *Zanclea* specimens studied by Fontana et al. (2012) (Montano et al. 2015a). However, no sequences are currently available for *Z. gilii* and *Z. margaritae*.

Considering that the diversity of this genus could be underestimated due to the difficulty of morphological identification (i.e. presence of cryptic species), molecular techniques, as part of an 'integrated taxonomy' approach (Dayrat 2005), may be very useful. For instance, DNA taxonomy techniques offer fast, objective, and repeatable means to assess species boundaries (Fontaneto et al. 2015). Particularly, three methods are emerging as valuable tools for species delimitations, namely the Automatic Barcode Gap Discovery (ABGD) (Puillandre et al. 2012), the Poisson Tree Processes (PTP) (Zhang et al. 2013), and the Generalised Mixed Yule Coalescent (GMYC) (Pons et al. 2006, Fujisawa and Barraclough 2013). However, when used alone,

these techniques do not qualify for character-based species descriptions, as requested by the International Code of Zoological Nomenclature (ICZN, 1999, Article 13.1.1). Character-based approaches, such as the Characteristic Attribute Organization System (CAOS), could therefore be used as tools to establish diagnostic characters from DNA sequences to be directly used in species descriptions (Sarkar et al. 2008, Zou et al. 2011, Jörger and Schrödl 2013), and this approach has already allowed taxonomists to formally describe some cryptic taxa identified with molecular methods at both the species and the genus level (e.g. Churchill et al. (2014), Johnson et al. (2015), Shipman and Gosliner (2015), Zielske and Haase (2015), Scarpa et al. (2016)). However, in most cases, cryptic species remain nameless because of the lack of additional evidences other than molecular data and, when named, the use of DNA alone could potentially lead to the establishment of a parallel nomenclature, especially if only few molecular markers are investigated. To prevent that, Morard et al. (2016) proposed a novel nomenclatural system to be applied to taxa showing a structured genetic diversity occurring below the level of morphospecies, viz. cryptic species. This method allows a reliable association of genetic units to any other type of data without creating taxonomical confusion, and permitting their inclusion in future researches, such as biodiversity assessments, endemism studies or conservation efforts. Moreover, this system easily allows the transfer of a given genetic group to formal nomenclature, as soon as it is diagnosed with other lines of evidence, such as new morphological characters or ecological information.

Herein, about 250 colonies of *Zanclea* associated with 30 scleractinian genera from several localities were analysed. The morphological and genetic diversity, the phylogenetic relationships, the species boundaries, the population structure, and the relationships with their hosts were investigated for *Z. gallii*, *Z. sango* and other unidentified *Zanclea* specimens by morphological assessments and by sequencing three nuclear and three mitochondrial molecular markers.

2.3. MATERIAL AND METHODS

2.3.1. Sample Collection

The sampling was conducted between March 2014 and May 2017 in several localities including the Indian Ocean, the Pacific Ocean, The Red Sea, and the Caribbean Sea (Figure 2.1).

The presence of *Zanclea* on scleractinian corals was recorded qualitatively *in situ* and small fragments of corals with hydrozoans 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 placed under a stereomicroscope. Afterwards, they were immediately preserved in 95 % ethanol for molecular analyses and fixed in 10 % formalin for morphological studies. Coral fragments were then submerged in a sodium hypochlorite solution in order to remove tissues and organic matter for scanning electron microscopy (SEM) studies. Additional portions of the colonies were immediately placed in outdoor tanks, and were subsequently cultured in small bowls through feeding *Artemia* nauplii to the *Zanclea* polyps in order to observe the release of medusae. Thereafter, the medusae were maintained in small bowls at ambient temperature and fed *Artemia* nauplii. The water was

replaced every day, two hours after feeding. The reared medusae were observed on a daily basis, and some medusae were fixed in 10% formalin.

2.3.2. Morphological Analyses

Morphological observations, pictures and measurements of the polyps, medusae and nematocysts were mainly performed using living specimens. Underwater photographs of *Zanclea*-coral associations were taken using a Canon G11 camera in a Canon WP-DC 34 underwater housing. Microphotographs of hydroids, medusae, and nematocysts were taken using a Leica EZ4 D stereomicroscope and a Zeiss Axioskop 40 microscope both equipped with a Nikon AW 100 camera and ocular micrometrics. For SEM analyses, bleached portions of coral colonies with hydroids were sputter-coated with gold-palladium in a Balzer Union evaporator and examined using a Philips XL20 scanning electron microscope.

All hydroids (except for *Zanclea gallii* and *Zanclea sango*) were identified to genus level according to Bouillon et al. (2006), while the scleractinian hosts were identified to genus level according to Veron (2000) and updated taxonomic classifications: Acroporidae Verril 1902 (Wallace et al. 2007), Agariciidae Gray 1847 (Terraneo et al. 2017), Coscinaraeidae Benzoni, Arrigoni, Stefani & Stolarski 2012 (Benzoni et al. 2012), Dendrophylliidae Gray 1847 (Arrigoni et al. 2014a), Fungiidae Dana 1846 (Gittenberger et al. 2011), Lobophylliidae Dai & Horng 2009 (Budd et al. 2012, Arrigoni et al. 2014b), Merulinidae Verril 1865 (Budd et al. 2012, Huang et al. 2014a, Huang et al. 2014b), Psammocoridae Chevalier & Beauvais 1987 (Benzoni et al. 2007), Pocilloporidae Koby 1890 (Kitahara et al. 2010), and Poritidae Gray 1842 (Kitano et al. 2014).

2.3.3. Molecular Analyses

The total genomic DNA of ethanol-fixed *Zanclea* samples from 28 scleractinian genera was extracted following a protocol modified from Zietara et al. (2000). Six 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 (*COX1*), iii) a ~700 bp portion of the mitochondrial cytochrome *c* oxidase subunit III gene (*COX3*), iv) a ~1700 bp portion of the nuclear *18S* ribosomal DNA gene (*18S rRNA*), v) a ~1700 bp portion of the nuclear *28S* ribosomal DNA gene (*28S rRNA*), and vi) a ~700 bp portion of the nuclear internal transcribed spacer ribosomal region (*ITS*). *16S*, *COX3*, *28S*, and *ITS* regions were amplified using hydrozoan-specific primers and the protocols proposed by Cunningham and Buss (1993), Peña-Cantero and Sentandreu (2017), Maggioni et al. (2016), and Fontana et al. (2012), respectively. *COX1* and *18S* 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° for 60 min, followed by 85° for 15 min and then directly sequenced in forward and reverse directions using an ABI 3730xl DNA Analyzer (Applied Biosystems). The obtained chromatograms were visually checked and assembled using Sequencher 4.1.4 (Gene Codes). *COX1* and *COX3* sequences were translated in Geneious 6.1.6 (Drummond et al. 2010), in order to check for the presence of stop codons. Sequences of each marker were aligned with MAFFT v. 7.110 (Katoh and Standley 2013) using the E-INS-i

option and *16S*, *18S*, *28S*, and *ITS* alignments were run through Gblocks (Castresana 2000, Talavera and Castresana 2007) using the default 'less stringent' settings in order to remove ambiguously aligned regions.

2.3.4. Genetic Diversity and Species Delimitation

Phylogenetic inference analyses were performed for all single locus datasets, for the nuclear dataset and the mitochondrial datasets using Bayesian inference (BI) and maximum likelihood (ML). Appropriate partition schemes and models were determined using PartitionFinder 1.1.1 (Lanfear et al. 2012) by means of the Akaike Information Criterion (AIC). BI analyses were performed using MrBayes 3.2 (Ronquist et al. 2012). Four parallel Markov Chain Monte Carlo runs (MCMC) were run for 10^7 generations for each dataset. Trees were sampled every 100^{th} generation and burn-in was set to 25%, based on checking the parameter estimates and convergence using Tracer 1.6 (Rambaut et al. 2014). Maximum likelihood trees were built with Garli 2.01 (Zwickl 2006) and read into the SumTrees 4.0.0 program in the DendroPy 4.0.0 package (Sukumaran and Holder 2010) to calculate non-parametric bootstrap support (BS) values from 1000 replicates, each based on five heuristic search replicates, and to map them on the best ML tree. Mitochondrial single-locus ultrametric trees were built for species delimitation analyses using Beast 1.8.2 (Drummond et al. 2012), using a relaxed log-normal clock with a coalescent tree prior: MCMC were run for $5x10^7$ generations, sampling every 1000 generations, chain convergence was assessed using Tracer 1.6 (Rambaut et al. 2014), and the consensus trees (with 25% burn-in) were built with TreeAnnotator 1.7 (Rambaut and Drummond 2013).

Genetic distances (uncorrected p-distance, 1000 bootstrap) within and among *Zanclea* molecular lineages were computed for each separated molecular locus using MEGA 6 (Tamura et al. 2013).

To determine molecular species in the single-locus mitochondrial datasets, three independent species delimitation approaches were used, the ABGD, PTP, and GMYC. ABGD analyses (Puillandre et al. 2012) were run on the web server http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html. The alignments were imported in MEGA 6.0 (Tamura et al. 2013) to compute matrices of pairwise genetic distances using the Kimura 2-parameter (K2P), p-distance, and Jukes-Cantor (JC69), and parameters were set as follows: Pmin = 0.001, Pmax = 0.05, Steps = 50, X = 1.5, and Nb bins = 20. PTP and bPTP analyses (Zhang et al. 2013) were performed on the web server http://species.h-its.org/ptp/, using the Bayesian trees. PTP analyses were run for $5x10^5$ MCMC generations, with thinning value = 100 and burn-in = 0.25. Clusters with a probability ≥ 0.9 were considered as corresponding to species. Finally, ultrametric trees were used to perform single-threshold (stGMYC) (Pons et al. 2006), multiple-threshold (mtGMYC) (Monaghan et al. 2009), and Bayesian (bGMYC) (Reid and Carstens 2012) GMYC analyses in R 3.1.3 (R Core Team, 2013) using the packages 'Splits' (Ezard et al. 2009), 'Ape' (Paradis et al. 2004) and 'bGMYC' (Reid and Carstens 2012). For bGMYC analysis, clusters with a probability ≥ 0.9 were considered as successfully delimited species.

CAOS software was used to identify diagnostic nucleotides for the newly recovered species (Sarkar et al. 2002, Sarkar et al. 2008, Bergmann et al. 2009), following the instructions of Jörger and Schrödl (2013). Only single pure character attributes were considered as diagnostic characters, which are single nucleotides present in all members of a clade identified as a species, but absent in members of other clades (Jörger and Schrödl 2014).

The nomenclatural system recently proposed by Morard et al. (2016) was then used in order to name the recovered cryptic clades. This method is based on the definition of basetypes and the use of their hierarchical phylogenetic structure to define levels of divergence below that of morphospecies. A basetype is defined as a specific DNA substitution pattern observed within a single marker gene, whereas a basegroup is a set of basetypes and constitute the lowest molecular operational taxonomic unit (MOTU) level. If a gene exists in a unique version, a basegroup will contain only one basetype, otherwise it will contain all pairs of basetypes observed to co-occur in one individual. The genetic variability between the basegroup and the morphospecies is then used to identify intermediate levels at different degrees of divergence, and any of these levels could be theoretically considered a species hypothesis. Similarly to Morard et al. (2016), it is here proposed to define basetypes in hydrozoans by using sequence patterns in a widely used barcode gene in the group, namely the *16S rRNA*.

For each recovered species, single-locus median-joining haplotype networks were built using PopArt 1.7 (Leigh and Bryant 2015) and colours were assigned according to geographic provenience.

For the three mitochondrial markers, the number of haplotypes (H), gene diversity (h), nucleotide diversity (π), and statistics for neutral sequence evolution (Tajima's D and Fu's Fs) were calculated for species and populations using Arlequin 3.5.1.2 (Excoffier and Lischer 2010). Genetic differentiation among the detected species and populations was tested using analysis of molecular variance (AMOVA), and by pairwise *Fst*, in Arlequin 3.5.1.2.

2.3.5. Testing Coevolution Between *Zanclea* and Corals

A tanglegram of associations among terminals of the *Zanclea* and host phylogenies was assembled using TreeMap 3 (Charleston and Robertson 2002) and corals phylogeny was manually assembled using most updated available information in literature (Fukami et al. 2008, Kitahara et al. 2010, Gittenberger et al. 2011, Arrigoni et al. 2017). To evaluate a possible congruence between the *Zanclea* and hosts phylogenies, the program Jane 4.0 (Conow et al. 2010) was used. A cost to each evolutionary event was assigned as follows: cospeciation (0), duplication (1), duplication-host switching (2), loss (1), and failure to diverge (1). Statistical analyses were performed by comparing the optimal (minimum) costs found for the *Zanclea*-hosts dataset against randomized datasets (Cruaud et al. 2012). The following settings were used (stats mode): 100 generations, population size 500, and sample size 100. All other settings were left unchanged.

Ancestral state reconstructions were performed for the characters perisarc (present-absent), eurytele capsules (present-absent), state of the colony (polymorphic-monomorphic, where polymorphic means with dactylozooids other than gono-gastrozooids) and host specificity (specific-generalist), in order to reconstruct the possible morphological and ecological features of the most recent common ancestor of coral-associated *Zanclea*. Stochastic mapping (Huelsenbeck et al. 2003) was used to map probable realisations of the evolution of the considered characters on the coral-associated *Zanclea* tree. The analyses were carried out using the 'make.simmap' function available in the R package 'Phytools' (Revell 2012). The 'equal rate' model was used to evolve the interactions along the phylogenetic trees, and 1000 stochastic mappings replicates were

conducted for each analysis. Simulations were then summarised in density plots (Revell 2013) with the colour of edges indicating the posterior probability of each state of character.

2.4. RESULTS

Throughout the surveys conducted from 2014 to 2017, *Zanclea* colonies associated with corals were collected from Faafu Atoll (Maldives), Eilat (Israel), Dahab (Egypt), Thuwal and Farasan Banks (Saudi Arabia), Bali (Indonesia), Ko Tao (Thailand), and Sint Eustatius (Dutch Caribbean) (Figure 2.1). Maldives and Red Sea were extensively investigated through several field surveys from 2014 to 2017. Caribbean Sea was explored in several localities (Panama, Costa Rica, Curaçao, and Sint Eustatius) but only two *Zanclea* colonies were found around Sint Eustatius. Finally, Indonesia and Thailand were only briefly explored and just few samples were collected. The hydrozoans were found in association with 30 coral genera, belonging to at least 10 families, namely Acroporidae: *Acropora*, *Montipora* de Blainville 1830; Poritidae: *Porites* Link 1807, *Goniopora* de Blainville 1830; Dendrophyllidae: *Turbinaria* Lamouroux 1825; Agariciidae: *Pavona*, *Gardineroseris* Scheer & Pillai 1974, *Leptoseris* Milne, Edwards & Haime 1849, *Pachyseris* Milne, Edwards & Haime 1849; Lobophylliidae: *Lobophyllia* de Blainville 1830, *Symphyllia* Milne, Edwards & Haime 1848; Merulinidae: *Dipsastrea* de Blainville 1830, *Favites* Link 1807, *Platygyra* Ehrenberg 1834, *Goniastrea* Milne, Edwards & Haime 1848, *Echinopora* Lamarck 1816, *Orbicella* Dana 1846, *Cyphastrea* Milne, Edwards & Haime 1848, *Phymastrea* Milne, Edwards & Haime 1848; Pocilloporidae: *Stylocoeniella* Yabe & Sugiyama 1935; Psammocoridae: *Psammocora*; Coscinaraeidae: *Coscinaraea* Milne, Edwards & Haime 1848; Fungiidae: *Fungia* Lamarck 1801, *Halomitra* Dana 1846, *Podabacia* Milne, Edwards & Haime 1848, *Cycloseris* Milne, Edwards & Haime 1848, *Lithophyllon* Rehberg 1892, *Danafungia* Wells 1966, *Pleuractis* Verril 1864; Scleractinia *incertae sedis*: *Leptastrea* Milne, Edwards & Haime 1848 (Figure 2.2). Colonies were mostly collected from shallow waters (0-20 m deep), but in some cases, also deeper associations were found (*Zanclea* on *Montipora* in Dahab: 38 m deep; *Zanclea* on *Orbicella* in Sint Eustatius: 41 m deep).

2.4.1. Morphological analyses

The characters considered in the morphological study of polyps correspond to the polymorphism of the colony (Figure 2.3A), the number and position of medusa buds (Figure 2.3A-C), the size of polyps, the number of tentacles, the size of capitula (Figure 2.3D, E), the perisarc (Figure 2.3 F, G), the cnidome (Figure 2.3H-J), and the presence of a calcareous overgrowth of coral skeleton around the pedicel (Figure 2.3K, L). Medusae were studied in their size, tentacle length (Figure 2.4A), nematocyst type and distribution (Figure 2.4B-F), and cnidophores shape and number (Figure 2.4G).

Morphological analyses allowed the identification of three different morphotypes. Two morphotypes correspond to the already described species *Zanclea sango* and *Zanclea gallii*, whereas a third morphology represents a new type of coral-associated *Zanclea*. These three morphologies are described in the following ‘Systematics’ paragraph.

2.4.1.1. Systematics

Family Zancleidae Russel 1953

Genus *Zanclea* Gegenbaur 1857

Zanclea sango Hirose & Hirose 2011

<u>*Polyp*</u>: Polymorphic colonies comprising gono-gastrozooids and dactylozooids, and associated with the scleractinians genera *Goniopora, Turbinaria, Pavona, Gardineroseris, Leptoseris, Pachyseris, Lobophyllia, Symphyllia, Dipsastrea, Favites, Platygyra, Goniastrea, Echinopora, Orbicella, Cyphastrea, Phymastrea, Stylocoeniella, Psammocora, Coscinarea, Fungia, Halomitra, Podabacia, Cycloseris, Litophyllon, Danafungia, Pleuractis,* and *Leptastrea*. Hydrorhiza surrounded by a thin perisarc, growing in-between coral tissue and skeleton, and with the perisarc stopping at the base of the hydranth. Polyps arising from the coral surface and mainly scattered on corallite edges. Coral skeleton overgrowing the base of hydranths and forming a cylindrical tube up to 335 μm high and 70 μm wide. Cylindrical gastro-gonozooids (up to 0.9 mm high) with a whorl of 4-6 oral capitate tentacles (diameter of the capitula 40-45 μm), and 12-21 aboral capitate tentacles scattered along the hydranth body (diameter of the capitula 25-35 μm). Extensible dactylozooids (up to 2 mm high) with a globular apex rich in glandular cells lacking tentacles and hypostome. Medusa buds arising in groups of 2-4 from the basal portion of the hydranths or short blastostyles.

<u>*Medusa*</u>: Bell spherical (700-750 μm in diameter), with a cylindrical manubrium spanning one-third of the subumbrellar cavity (250-270 μm). Four perradial nematocyst pouches running along the exumbrella up to a half of its height, and with a marginal bulb at the base. Two small bulbs with no tentacles and two large, triangular bulbs bearing tentacles with 30-40 oval cnidophores. Each cnidophore containing 2-3 nematocysts.

<u>*Cnidome*</u>: i) Large stenoteles (11-15 x 10-14 μm) in capitula, dactylozooids, blastostyles, and nematocyst pouches. ii) Small stenoteles (7-10 x 6-8 μm) in capitula, dactylozooids, blastostyles, manubrium, and tentacular bulbs. iii) Apotrichous macrobasic euryteles (18-21 x 7-9 μm; discharged shaft: 145-155 μm) in hypostome and base of gastrozooids and gono-gastrozooids, in the apex and base of dactylozooids, and in the nematocyst pouches. iv) Bean-shaped apotrichous macrobasic euryteles (8 x 5 μm; discharged shaft: 35-40 μm) in cnidophores.

Zanclea gallii Montano, Maggioni & Puce 2014

<u>*Polyp*</u>: Polymorphic colonies living in association with scleractinians belonging to *Acropora* species. Gono-gastrozooids arising from the coral surface between the corallites or frequently on the corallites (often close to the *Acropora* polyps). Hydrorhiza lacking a perisarc and growing inside the coral, at the interface between and in direct contact with coral tissue and skeleton. Host producing a distinct collar-like tissue elevation at the base of hydroid polyps. No evident coral skeleton modification present, no cylindrical calcareous tube covering the base of hydranths. Gono-gastrozooids cylindrical (up to 1.1 mm high), with hypostome surrounded by 4-6 oral capitate tentacles (diameter of the capitula 50-55 μm). Hydranth body surrounded by 14-30 aboral capitate

tentacles (diameter of the capitula 40-45 μm). Contractile dactylozooids with thin hydranth body and a globular apex without tentacles, rarely present and exclusively observed in situ. Medusa buds arising in groups of 2-4 from the basal portion of the gono-gastrozooids and laterally or apically from short blastostyles.

Medusa: Spherical (0.8-1.1 mm in diameter) with a cylindrical manubrium with a length from one-fourth to one-third of the subumbrellar cavity (150-350 μm). Four perradial nematocyst pouches extending along the exumbrella. Two pouches short and placed above marginal bulbs without tentaces. Two pouches elongated and above large bulbs bearing tentacles. Tentacles armed with up to 60 cnidophores, each containing 3-5 nematocysts.

Cnidome: i) Large stenoteles (11-15 x 10-14 μm) in capitula, hydranth body (rare) hydrorhiza, blastostyles, and nematocyst pouches. ii) Small stenoteles (6-10 x 5-9 μm) in capitula, hydranth body (rare), hydrorhiza, blastostyles, and manubrium. iii) Bean-shaped apotrichous macrobasic euryteles (8 x 4-5 μm; discharged shaft: 35-40 μm) in cnidophores, bulbs, and circular canal.

Zanclea intermedia sp. nov.

Polyp: Colonies associated with the scleractinian genera *Porites* and *Montipora*. Hydrorhiza surrounded by a thin perisarc, growing under coral tissue and over coral skeleton, and with the perisarc stopping at the base of the hydranth. Gono-gastrozooids arising from the coral surface and randomly distributed on the coral coenosarc. Coral skeleton overgrowing the base of hydranths and forming cylindrical tubes up to 135 μm high and 65 μm wide. Gono-gastrozooids (up to 1 mm high) with a whorl of 5-6 oral capitate tentacles (diameter of the capitula 45-60 μm), and 26-30 aboral capitate tentacles scattered along the hydranth body (diameter of the capitula 35-50 μm). No dactylozooids observed. Medusa buds arising in groups of 1-5 from the basal portion of the hydranths or short blastostyles.

Medusa: Bell spherical (650-800 μm in diameter) with a cylindrical manubrium reaching one fourth of the subumbrellar cavity in lenght (160-200 μm). Four perradial nematocyst pouches extending along the exumbrella, ending in two large bulbs bearing tentacles and in two small bulbs without tentacles. Tentacles armed with up to 60 oval cnidophores, each containing 2-4 nematocysts.

Cnidome: i) Large stenoteles (12-15 x 10-13 μm) in capitula, hydrorhiza, blastostyles, nematocyst pouches, and bulbs (rare). ii) Small stenoteles (6-8 x 8-9 μm) in capitula, hydrorhiza, blastostyles, and manubrium. iii) Bean-shaped apotrichous macrobasic euryteles (7-8 x 3-4 μm; discharged shaft: 35-40 μm) in cnidophores, bulbs, and circular canal.

Etymology: The specific name derives from the fact that this species has morphological features shared with both *Z. sango* and *Z. gallii*: it shares with the first the presence of a perisarc around the hydrorhiza, and with the second the absence of eurytele capsules in hydranths.

2.4.2. Molecular analyses

2.4.2.1. Phylogenetic analyses and genetic distances

The total genomic DNA of the ethanol-fixed *Zanclea* samples was successfully extracted from colonies associated with 28 coral hosts (DNA extractions from specimens associated with *Lithophyllon* and *Lobophyllia* were unsuccessful), and six molecular markers were amplified for a total number of 950 sequences (*16S*: n = 240; *COX1*: n = 207; *COX3*: n = 203; *18S*: n = 100; *28S*: n = 100; *ITS*: n = 100). The total alignments of the *16S*, *COX1*, *COX3*, *18S*, *28S*, and *ITS* datasets were 565, 645, 650, 1680, 1607 and 545 bp long, respectively. PartitionFinder found the following partition scheme and models for the mitochondrial dataset: *16S* (GTR + G + I), *COX1*_pos1_2 *COX3*_pos1_2 (GTR + G + I), *COX1*_pos3 *COX3*_pos3 (HKY + G + I); and for the nuclear dataset: *18S 28S* (GTR + G + I), *ITS* (HKY + G + I).

Phylogenetic trees obtained from BI and ML analyses were similar and, therefore, only the Bayesian topology with significant branch supports (Bayesian posterior probability (BPP) > 0.95 and maximum likelihood bootstrapping (BS) > 75) indicated by white circles is shown in Figure 2.5A. The single- and multi-locus phylogenetic analyses of the mitochondrial dataset gave similar results, and only the concatenated analysis is shown, representing the best supported phylogenetic hypothesis (Figure 2.5A). Contrarily, nuclear markers were not able to discriminate among the different molecular lineages, due to their low variability (*18S*: π = 0.0001; *28S*: π = 0.0004; ITS: π = 0.002), and were therefore not included in the subsequent analyses.

Coral-associated *Zanclea* were rooted with other representative of the superfamily Zancleida (*Cladocoryne haddoni* Kirkpatrick 1890, *Velella velella* (Linnaeus 1758), and *Asyncoryne ryniensis* Warren 1908), resulting in a monophyletic clade with maximum statistical support (BPP = 1, BS = 100). Seven well-supported monophyletic lineages are recovered (Clades I, II, III, IVa, IVb, V, and VI) and these clades show different levels of host-specificity (Figure 2.5A). Hydroids belonging to Clade I (BPP = 1, BS = 100) are associated with *Goniastrea*, *Cyphastrea*, and *Phymastrea* from Maldives, and with *Gardineroseris* from Maldives and Red Sea and they are sister of the lineage composed of Clade II and III. Hydrozoans belonging to this clade were morphologically identified as *Zanclea sango*. Clade II (BPP = 1, BS = 95) and III (BPP = 1, BS = 100) are specifically associated with *Montipora* and *Porites*, respectively, and can be found in Maldives, Red Sea and also Taiwan (only Clade II). Their morphological identification corresponds to *Zanclea intermedia*. The remaining clades form a cohesive lineage well separated from the group composed of Clade I, II, and III. Specifically, the reciprocally monophyletic Clade IVa (BPP = 1, BS = 97) and IVb (BPP = 0.99, BS = 95) are associated with *Acropora* from several localities and are morphologically identical, being identified as *Zanclea gallii*: the first one can be found exclusively in the Red Sea, whereas the second one is found in Maldives, Red Sea, Indonesia, Taiwan, and Australia. Clade V (BPP = 0.99, BS = 92) is composed of hydrozoans associated with *Pavona* and collected in the Maldives, Red Sea, and Thailand. Finally, Clade VI (BPP = 1, BS = 100) is composed of hydrozoans associated with 22 coral genera from Maldives and Red Sea, including *Goniastrea*, *Cyphastrea*, and *Pavona*, which also host hydrozoans belonging to Clade I and V. Both Clade V and VI are morphologically identified as *Zanclea sango*.

Genetic distances are higher in the *COX3* dataset, followed by *COX1* and *16S* datasets (Table 2.1A, B, C). Within-clade genetic distances are generally low, ranging from 0.0 ± 0.0 % in the *16S* dataset to 1.6 ± 0.3 % in the *COX3* dataset. Inter-clade genetic distances are high for all markers and range from 3.2 ± 0.8 % in the *16S* dataset to 11.3 ± 1.2 % in the *COX3* dataset, with mean values of 3.1 ± 0.5 %, 4.4 ± 0.5 %, and 5.5 ± 0.5 for *16S*, *COX1*, and *COX3*, respectively.

2.4.2.2. Species delimitation

Species delimitation outputs were congruent for single- and multi-locus mitochondrial datasets and all recovered seven main lineages ascribable to independent molecular species and corresponding to the seven well-supported clades found with the phylogenetic analyses (Figure 2.6, Table 2.2). In some cases, some of the methods recovered a further subdivision of the clades, but only the clusters recovered in all the analyses are here considered as successfully delimited species (Table 2.2). The three morphospecies are therefore to be considered as species complexes (Figure 2.6): *Zanclea gallii* is composed of two *Acropora*-associated species; *Zanclea intermedia* is splitted in two species, one associated with *Porites* and one with *Montipora*; hydrozoans with a *Zanclea sango* morphology are not monophyletic and can be split in three further species, one associated with *Pavona* and two associated with a multitude of hosts. Re-analysis of morphological data after the molecular species definition did not allow to detect differences among morphologically identical cryptic species. CAOS software found several specific diagnostic positions in the three mitochondrial markers (Table 2.3) and each molecular lineage was named following the molecular nomenclature proposed by Morard et al. (2016) (Table 2.2). Clade I is now called *Zanclea intermedia* I, Clade II is *Zanclea intermedia* II, Clade III is *Zanclea intermedia* III, Clade IVa is *Zanclea gallii* II, Clade IVb is *Zanclea gallii* I, Clade V is *Zanclea sango* I, Clade VI is *Zanclea sango* II (for a complete list of the names of each clade and sub-clade see Figure 2.S1).

2.4.2.3. Haplotype networks

Median-joining haplotype networks for each species are shown in Figure 2.5B, C, D. Number of haplotypes of each population and species for each marker are summarised in Table 2.4. Overall, *COX1* sequences are characterized by a higher number of haplotypes (H = 42) than *16S* (H = 34) and *COX3* (H = 33) sequences, the latter two having a comparable H value. Moreover, *COX1* and *COX3* haplotypes are separated by a two to four times higher number of substitutions compared to *16S* haplotypes. However, all networks are congruent with mitochondrial phylogenetic reconstructions and they are similar between each other, with no haplotypes shared between representatives of two or more clades. According to haplotype networks, populations from the Red Sea and Maldives are quite well separated and show private haplotypes in *Zanclea gallii* I, *Zanclea intermedia* II, and *Zanclea intermedia* III (only in *COX1* and *COX3* sequences). *Zanclea gallii* I shows a further subdivision between Indian and Pacific populations, whereas in *Z. sango* I, the population from Thailand is clearly separated from specimens from Maldives and Red Sea. The two generalist species (i.e. *Zanclea intermedia* I and *Z. sango* II) do not show a clear geographical diversification.

2.4.2.4. Population genetics

Regarding population genetics indices, *16S* and *COX1* show similar results (Table 2.4A, B), whereas *COX3* dataset show slightly different results (Table 2.4C). Gene diversity (h) is higher in *Zanclea gallii* I and II for all markers, and high h values are also shown by *Zanclea intermedia* II and *Zanclea sango* II from the Red Sea. Other populations generally have intermediate to low gene diversity levels. In general, populations of coral-associated *Zanclea* do not show significant departures from neutrality (P > 0.05). However, the population of *Z. sango* II in the Red Sea has significant values of Fu's Fs for all markers (*16S* = -1,762; *COX1* = -2,135; *COX3* = -1,644) and of Tajima's D for *16S* (-6,099) (Table 2.4A, B, C).

Whole-datasets AMOVA analyses confirmed that most of the genetic variation (> 90 % for all markers) is explained by molecular species grouping (Table 2.5A, B, C). Regarding single-species AMOVAs, only in few cases variation among population accounted for most of the genetic variation. Specifically, in *Z. intermedia* II 66, 82, and 74 % of the *16S*, *COX1*, and *COX3* genetic variation, respectively is explained by inter-population differentiation (Table 2.5A, B, C). In *Z. gallii* I, intermediate levels of variation can be attributed to differentiation among populations (*16S*: 62 %; *COX3*: 57 %) (Table 2.5A, C), whereas in *Zanclea intermedia* I and *Zanclea intermedia* III, inter-population variation accounted for most of the genetic diversity only in *COX1* (85 %) and *COX3* (89 %) datasets (Table 2.5B, C).

In terms of pairwise population differentiation, values are generally high and significant for populations belonging to different molecular species (Table 2.6A, B, C). Regarding intra-specific population comparisons, fixation index values are high and significant for *Z. intermedia* II, *Z. intermedia* III (not in *16S* dataset), *Z. sango* II (comparisons between populations from Caribbean Sea and Red Sea or Indo Pacific), and *Z. gallii* I (not in *COX1* dataset) (Table 2.6A, B, C).

2.4.2.5 Testing Coevolution Between Zanclea and Corals

Regarding coevolutionary analyses, thirty-one associations were mapped on the tanglegram for seven *Zanclea* terminals and 28 host terminals (Figure 2.7). The tanglegram shows that the generalist *Zanclea* lineages are associated with representatives of both the two main scleractinian clades ('*Complexa'* and '*Robusta'*) (Romano and Palumbi 1996), whereas host-specific *Zanclea* lineages are associated with corals exclusively belonging to the '*Complexa'* clade. Thus, the latter coral clade hosts the highest diversity of *Zanclea* hydrozoans, with all *Zanclea* lineages found in association with one or more genera, whereas only two *Zanclea* lineages are associated with the '*Robusta'* clade. Jane analyses under default settings led to 3205 reconstructions (cost = 47) and the mean costs were estimated as 106.9 ± 6.1 for random associations and 108.9 ± 7.4 for random parasite topology. Failures to diverge was most used (25), followed by 16 losses, 2 duplications with host switch, 2 cospeciation events, and 2 duplications (Figure 2.S2).

Ancestral state reconstructions show that the ancestral coral-associated *Zanclea* was likely to be specifically associated with a coral taxon and that host-generalism independently arose two times, in *Zanclea sango* II and *Zanclea intermedia* I (Figure 2.8A). The ancestral *Zanclea* associated with corals also likely had hydrorhiza and pedicels covered by chitinous perisarc and perisarc was lost only in the *Acropora*-associated clade (Figure

2.8B). Colony polymorphism seems to be an ancestral state for these hydrozoans (Figure 2.8C). Finally, euryteles were lost or reobtained multiple times but stochastic mapping was not able to map on the three the ancestral presence or absence of euryteles with high probability (Figure 2.8D).

2.5. DISCUSSION

Throughout the field surveys conducted from 2014 to 2017, the host range of coral-associated *Zanclea* was increased to a total of 33 coral genera (Table 2.7), with seven new host recorded (*Turbinaria*, *Coscinaraea*, *Gardineroseris*, *Pachyseris*, *Lobophyllia*, *Phymastrea*, and *Stylocoeniella*). Moreover, new localities were added to the distribution of this association, including central Red Sea (Saudi Arabia), Thailand, and Caribbean Sea (Sint Eustatius) (Table 2.7). Therefore, coral-associated *Zanclea* is now confirmed to be a widespread group, living in the Indian, Pacific and Atlantic Ocean, as well as in the Red Sea. The Maldives and the Red Sea were the most studied localities in this study and hosted high numbers of coral genera associated with hydrozoans (Maldives: N = 26; Red Sea: N = 20). The Caribbean Sea was extensively investigated (Panama, Costa Rica, Sint Eustatius, and Curaçao) but only two colonies were found around Sint Eustatius. Other localities (Indonesia and Thailand) were less investigated during this study and further surveys will likely increase the number of coral hosts. Pantos and Bythell (2010) and Fontana et al. (2012) also inspected other corals for the presence of associated hydrozoans (*Seriatopora* Lamarck 1816, *Stylophora* Schweigger 1820, *Pocillopora* Lamarck 1816, *Porites*, *Montipora*, *Goniastrea*, *Leptoria* Milne Edwards & Haime 1848, *Dipsastrea*, *Galaxea* Oken 1815, *Pavona*, and *Echinophyllia* Klunzinger 1879), but they found *Zanclea* colonies only on Acroporidae, and this could be due to the low number of investigated colonies (Pantos and Bythell 2010). Therefore, it is likely that additional scleractinian hosts will be found in areas that are rich in coral species, such as the Coral Triangle (Hoeksema 2007) and the South China Sea (Huang et al. 2015).

The results provided in this study currently represent the most comprehensive phylogenetic assessment of the coral-associated *Zanclea*, including specimens associated with almost all the known hosts and from most of the known localities. In addition to the commonly used DNA barcode for Hydrozoa, the *16S* gene, (Zheng et al. 2014), it is herein shown that the genes *COX1* and *COX3* allow the recognition of separate hidden lineages in agreement with *16S* data. Indeed, *COX1* and *COX3* turned out to be more variable than *16S*, having approximately two-four times more substitutions compared with *16S*. Therefore, the levels of divergence observed within *Zanclea* associated with scleractinians strongly encourage and support the use of both *COX1* and *COX3*, along with *16S* sequences in phylogenetic studies of these hydroids. This conclusion is consistent also with previous molecular works that successfully used *COX1* gene to evaluate the potential presence of cryptic species or intraspecific population subdivision for instance in *Plumularia setacea* Linnaeus 1758 (Schuchert 2014), *Obelia geniculata* Linnaeus 1758 (Govindarajan et al. 2005), and in the genus *Cordylophora* Allman 1844 (Folino-Rorem et al. 2009). According to the mitochondrial phylogenetic trees, all *Zanclea* specimens associated with scleractinians group together in a cohesive and monophyletic cluster. Moreover, they are characterised by a considerable genetic diversity that it is not fully represented by the morphological

diversity. Indeed, several lines of evidence provided by molecular phylogenetics, genetic distance comparisons, species delimitations, population genetics, and host preference data show that all the morphospecies are actually species complexes, composed of two or more independent species.

Specifically, morphology allowed the identification of only three morphospecies, namely *Zanclea sango*, *Zanclea gallii* and the newly described *Zanclea intermedia*, whereas molecular phylogenetic analyses recovered seven well-supported clades, which were confirmed to correspond to as many species through DNA taxonomy and population genetics analyses. Also comparisons of genetic distances were concordant in the assignment to independent species status to all molecular clades rather than considering these lineages to be the result of a strong population subdivision. Although we are far from the establishment of an appropriate and widely accepted genetic distance threshold to differentiate hydrozoan species using *16S* sequences, Moura et al. (2011) proposed a conservative maximum of 2% divergence for intraspecific sequence distance in the Sertulariidae Lamouroux 1812. In our *16S* dataset, as well as in the more variable *COX1* and *COX3*, all the intra-clade distances are under this value, while the inter-clade divergences exceed this conservative threshold in all the pairwise comparisons. Furthermore, comparable *16S* genetic distances revealed the existence of cryptic species for instance within *Cordylophora* (up to 6 %) (Folino-Rorem et al. 2009), *Nemertesia* Lamouroux 1812 (up to 4.8 %) (Moura et al. 2012b), *Stylactis* Allman 1864 (up to 6 %) (Miglietta et al. 2009), *Cryptolaria* Lusk 1857 (up to 2.2 %) (Moura et al. 2012a), and *Lafoea* Lamouroux 1812 (up to 5 %) (Moura et al. 2008).

Using the Characteristic Attribute Organization System, molecular characters for species identification are here provided for the seven lineages, and names are given to each molecular entity, thus easily allowing their inclusion in further studies. The names are to be intended as provisional, until further lines of evidence will allow the formal description of the cryptic species (Morard et al. 2016). With the addition of the previously unknown new and cryptic species, the number of species associated with corals is raised to nine. However, it is still not clear whether *Zanclea gallii* I and *Zanclea margaritae* are conspecific, since sequences 'suspected to belong to *Z. margaritae*' from Australia generated by Fontana et al. (2012) clustered with *Z. gallii* I. In addition, *Zanclea gilii* may be conspecific with one of the species with a *Z. sango*-like morphology. Indeed, Boero et al. (2000) did not mention anything about the presence of a perisarc in *Z. gilii*, and Hirose and Hirose (2011) interpreted this fact as a lack of chitinous structures in this species. Nevertheless, if *Z. gilii* has the hydrorhiza embedded in a perisarc, it would be morphologically identical to the *Z. sango* morphotype. Finally, the identity of the coral-associated *Zanclea* sp. with perisarc found by Millard and Bouillon (1974) in Mozambique cannot be elucidated due to missing information regarding the cnidome (stenoteles of two sizes and bean-shaped capsules measuring 13.8 x 6 μm).

All the species, with the exception of *Zanclea gallii* II, can be found in both Maldivian waters and the Red Sea, and *Zanclea sango* I, *Zanclea intermedia* II, and *Z. gallii* I are also found in the Pacific Ocean (Table 2.7). In this study, *Z. sango* I was found in strict association only with *Pavona* corals, whereas *Psammocora* was found to host only colonies of *Zanclea sango* II. Therefore, it is possible that the *Psammocora*-associated specimens described by Hirose and Hirose (2011) actually belong to *Z. sango* II, extending, if it is the case,

the distribution of this species to the Pacific Ocean. *Zanclea sango* II is the only species with a distribution extended to the Caribbean Sea, where it is nevertheless rare. Notably, these specimens from the Caribbean share the same haplotypes (for all markers) with colonies from the northern Red Sea associated with *Favites*. One of the possible interpretations of this fact is that the apparently small and rare population from the Caribbean could represent a potential introduction or recent distributional range extension. A similar situation is also observed in other hydrozoans, such as *Turritopsis* sp. 4 (*sensu* Miglietta and Lessios (2009)) from the Caribbean, which share similar haplotypes with a colony of *Turritopsis* McCrady 1857 from the Red Sea (own unpublished data). However, for a better understanding of this issue, further ecological and genetic surveys need to be carried out in several Atlantic, Indian, and Pacific localities, to have a better picture of the distribution of this group and to help tracking its possible routes of introduction. *Zanclea gallii* II is the only species exclusively found in the Red Sea and represents one of the three currently known *Acropora*-associated species. Two of these species (i.e. *Z. margaritae* and *Z. gallii* II) appear at this stage to have non-overlapping geographic distributions, whereas *Z. gallii* I has a distribution overlapping with both the two other species. The high diversity of *Acropora*-associated *Zanclea* might be at least partially explained by the ecological and evolutionary traits of *Acropora* corals. *Acropora* species are characterised by fast growth rates and are more prone to stressors (e.g. Marshall and Baird (2000), Loya et al. (2001), Montano et al. (2010), Furby et al. (2013)). However, this genus has been seen to constitute one of the major taxonomic components of recruits to settlement plates (Sammarco and Carleton 1981), to dominate in some cases coral assemblages recovering from damage (Pearson 1981) and to regenerate from fragments (Wallace 1985). These characteristics suggest an important early successional role for this genus, with some species demonstrating high population turnover rates (e.g. Guzner et al. (2007)). The ecological nature of the *Acropora* genus might therefore have influenced the speciation rate of its specifically associated organisms, such as *Zanclea* hydrozoans. Another possible explanation of the *Acropora*-related *Zanclea* diversity could refer to its biogeography, especially regarding *Z. gallii* II, being this species found exclusively in the Red Sea. The Red Sea is considered a biodiversity and endemicity hotspot (Hughes et al. 2002), is isolated from the Indian Ocean (Eshel et al. 1994) and is characterised by heterogeneous environmental conditions of both inner and bordering waters (Sofianos and Johns 2003, Raitsos et al. 2013). These unusual environmental conditions likely contributed to the high number of endemic species in the Red Sea (DiBattista et al. 2016) and might have played a role also in the speciation of *Z. gallii* II, even though further investigations in the southern Red Sea, the Gulf of Aden and nearby areas are needed to fully assess the endemicity of this species.

Population genetics analyses revealed that also some other *Zanclea* populations from the Red Sea are significantly separated from Indian and Pacific populations, and the geographical and environmental causes discussed above could have played a role also in these intra-specific population structures. Moreover, *Acropora* corals are not the only ones associated with more than one *Zanclea* species, since the genera *Pavona*, *Goniastrea*, and *Cyphastrea* were found in association with both the two generalist species. However, these corals never hosted the two species at the same time and in the same locality, since *Pavona* was found in association with *Z. sango* II only in the central Red Sea, where *Z. sango* I was not found, while *Goniastrea*

and *Cyphastrea* hosted *Z. sango* II in the Red Sea and *Zanclea. intermedia* I in the Maldives. This overlapping host use suggests that generalist coral-associated *Zanclea* species may compete for the host between each other and with other specifically associated species.

With the exception of *Z. sango* II and *Z. intermedia* I, all other coral-associated *Zanclea* species are specifically associated with a single coral genus, and this partially confirm the hypothesis proposed by Fontana et al. (2012), according to which *Zanclea* settle on coral belonging to a preferred genus. All the 'specific' species live in association with representatives of the '*Complexa'* clade, in particular with *Acropora, Montipora* (Acroporidae), *Porites* (Poritidae), and *Pavona* (Agariciidae) and, in general, this coral clade hosts the highest *Zanclea* diversity. Contrarily, the '*Robusta'* clade hosts only the two generalist *Zanclea* species. With some important exceptions, '*Robusta'* corals generally comprehend taxa with solid and heavily calcified skeletons that result from the solid construction of corallite walls, and colonies are mostly massive and foliose. On the other hand, '*Complexa'* corals tend to be less heavily calcified, and corallite walls and other skeletal elements are more porous, resulting in a relatively light, complex architecture (Romano and Palumbi 1996). The morphological features of '*Complexa'* corals may have facilitated the instauration of specific associations with *Zanclea* hydrozoans, since the hydrorhiza is in direct contact with coral skeleton, especially in perisarc-free species (Pantos and Hoegh-Guldberg 2011). Moreover, '*Complexa'* coral architectures (e.g. ramified, digitate, columnar, lamellar growth forms) are thought to increase coral symbiomes complexity (Gates and Ainsworth 2011) and this could facilitate the instauration of specific associations among *Zanclea*, corals, and other coral associates.

Concerning *Zanclea* morphological traits related to host specificity, Boero et al. (2000) and Puce et al. (2002) noted the importance of the presence or absence of a perisarc around the hydrorhiza and the monomorphic or polymorphic state of the colony. The authors suggested that ancestral species are predicted to be host generalists, monomorphic, and characterized by hydrorhiza covered by a perisarc, whereas advanced species that establish specific associations with host species should have lost their perisarc. An alternative hypothesis is that these traits, as the presence of macrobasic euryteles (Boero et al. 2000), instead of being a derived character, might be due to independent events of loss and acquisition of the related structure. The morphological and genetic evidences provided with this study show that perisarc and polymorphic colonies can be found in both generalist and specifically associated species. According to ancestral state reconstructions, the ancestral coral-associated *Zanclea* was already polymorphic and with the hydrorhiza covered by perisarc; the perisarc and colony polymorphism were subsequently lost in two independent events. The presence of euryteles is confirmed to be easily lost and regained in *Zanclea* hydrozoans, as already suggested by Boero et al. (2000). Finally, the ancestral reconstruction of the state 'host-specificity' suggests that the ancestral *Zanclea* was host-specific and that developed a generalist habit independently in *Z. sango* II and *Z. intermedia* I. Starting from an ancestral host-specific *Zanclea,* speciation may have occurred by both host shift and geographic isolation. For instance, in the *Acropora*-associated group, an allopatric speciation can be easily hypothesised, whereas in other cases a speciation by host shift seems a more reasonable hypothesis. Other studies have already demonstrated that this latter type of speciation can occur in coral-associated organisms

(Munday et al. 2004, Faucci et al. 2007, Tsang et al. 2009) and it has also been proposed that intra-specific competition may be a major driver of this kind of speciation, since corals not involved in the associations may represent a refuge from both intra- and inter-specific competition (Munday et al. 2004). Other causes of speciation by host-shift in coral associates, and in particular in organisms that settle on corals, may be linked to variation in coral microbiome or in larval receptors of coral symbionts. It has been demonstrated that bacterial cues are important drivers of larval settlement and metamorphosis in some hydrozoan species (e.g. Müller (1969), Edwards et al. (1987), Thomas et al. (1987)). For instance, settlement and metamorphosis of larvae of *Hydractinia echinata* (Fleming 1828) on shells inhabited by hermit crabs are mediated by cues produced by bacteria such as *Pseudoalteromonas espejiana* (Müller and Leitz 2002). Therefore, changes in planula receptors and/or in host microbiome may promote speciation by host-shift. Another type of speciation in symbiotic organisms is the co-speciation of hosts and symbionts, but this is probably not the case of coral-associated *Zanclea*. Indeed, even if Jane analysis found two possible co-speciation events, the divergence events among coral genera are likely to be older than the divergence events in coral-associated *Zanclea* (e.g. the divergence between the clades that later originated Acroporidae and Poritidae is estimated to have occurred around 400 millions years ago (Arrigoni et al. 2017)). Moreover, the divergence time of *Zanclea* species cannot be estimated at the moment, due to the absence of reliable fossils to time-calibrate the phylogenies. A possible solution could be to find traces of coral-associated *Zanclea* in coral fossils, looking, for instance, for the presence of skeletal modifications (i.e. skeletal overgrowth of polyp pedicels resulting in cylindrical tubes).

The assessment of the mode and tempo of diversification of coral-associated hydrozoans could also help in understanding when and how this group radiated. The hydrozoan-coral association has been documented only recently, with an increase of reports concerning its occurrence in the last few years (Pantos and Bythell 2010, Hirose and Hirose 2011, Fontana et al. 2012, Montano et al. 2013, Montano et al. 2014, Montano et al. 2015b). Thus, it is virtually impossible to establish whether this association has emerged in recent times, or whether it has simply be ignored in the past, possibly due to the small size of the hydrozoan polyps, which makes their detection difficult. However, the absence of previous data prevent from excluding a possible recent spread of this association in coral reefs, even if patterns of host specificity suggest the existence of a longstanding co-evolutionary history. Insights into the co-evolution of corals and hydrozoans could also be obtained through the understanding of the nature of this association. Different hypotheses have been proposed to explain this symbiosis, including mutualism and parasitism. The ability of hydrozoans to creep into coral tissues without triggering any immune reaction suggests that their relationship with corals is more intimate than an opportunistic epibiosis and Pantos and Bythell (2010) hypothesised mutualism related to increased protection of the host through the additional hydrozoan nematocysts and the hydroid activity that could remove detritus or pathogenic protozoans from its surface. This idea is also supported by the recent work of Montano et al. (2017), in which the authors showed that corals hosting *Zanclea* polyps are less susceptible to predation and diseases. Another outcome for the coral host might be related to an increased competitivity. Indeed, other *Zanclea* species associated with bryozoans are thought to increase the competitivity of their hosts, due to their presence also in peripheral parts of the host (Osman and Haughsness 1981, Ristedt and Schumacher, 1982).

This might be the case of *Zanclea* associated with massive and encrusting corals, since, in these cases, hydroids are often found in the periphery of coral colonies (personal observation). Contrarily, in branching corals, such as *Acropora*, hydrozoans are mostly found on branches (Montano et al. 2015a) and the main outcome for the coral host could be, in this case, defence from predation and diseases (Montano et al. 2017). On the other hand, the hydroid may benefit from food provided by the increased water flow and protective mucus on the coral surface. Also, a few zooxanthellae have been observed in the *Z. sango* I and *Z. margaritae* polyp coelenteron (Pantos and Bythell 2010, Hirose and Hirose 2011, Montano et al. 2015a), suggesting a trophic interaction between corals and hydroids. Alternatively, the relationship may be parasitic, based on the observed co-occurrence in few cases of bleaching and white syndrome with hydrozoans. For instance, both *Z. margaritae* and *Z. gallii* I were found on certain bleached *Acropora muricata* (Pantos and Bythell 2010, Montano et al. 2015a), but the sequence of cause and effect remains unknown.

Global change is now rapidly depleting coral communities at an unprecedented, fast rate (Hughes et al. 2010), and, recently, entire reefs have experienced severe mortality events (Hughes et al. 2017). It is therefore of imperative importance to fully characterise the potentially beneficial or detrital associates of scleractinian corals and, to do that, it is essential to integrate multidisciplinary studies of multiple organisms simultaneously. This will allow a better understanding of the functions and responses to stressors of the corals as single functional symbiotic systems (Gates and Ainsworth 2011), will help in the modelling aimed at better predicting climate change impacts on coral reefs and, eventually, in directing conservation efforts.

2.6. REFERENCES

Abouna S, Gonzalez-Rizzo S, Grimonprez A, Gros O (2015). First description of sulphur-oxidizing bacterial symbiosis in a cnidarian (Medusozoa) living in sulphidic shallow-water environments. *PloS One* 10: e0127625.

Arrigoni R, Kitano YF, Stolarski J, Hoeksema BW, Fukami H, Stefani F et al., Benzoni F (2014a) A phylogeny reconstruction of the Dendrophylliidae (Cnidaria, Scleractinia) based on molecular and micromorphological criteria, and its ecological implications. *Zoologica Scripta* 43: 661-688.

Arrigoni R, Terraneo TI, Galli P, Benzoni F (2014b) Lobophylliidae (Cnidaria, Scleractinia) reshuffled: pervasive non-monophyly at genus level. *Molecular Phylogenetics and Evolution* 73: 60-64.

Arrigoni R, Vacherie B, Benzoni F, Stefani F, Karsenti E, Jaillon O et al., Wincker P (2017) A new sequence data set of SSU rRNA gene for Scleractinia and its phylogenetic and ecological applications. *Molecular Ecology Resources* 17: 1054-1071.

Benzoni F, Arrigoni R, Stefani F, Stolarski J (2012) Systematics of the coral genus *Craterastrea* (Cnidaria, Anthozoa, Scleractinia) and description of a new family through combined morphological and molecular analyses. *Systematics and Biodiversity* 10: 417-433.

Benzoni F, Stefani F, Stolarski J, Pichon M, Mitta G, Galli P (2007) Debating phylogenetic relationships of the scleractinian *Psammocora*: molecular and morphological evidences. *Contributions to Zoology* 76: 35-54.

Bergmann T, Hadrys H, Breves G, Schierwater B (2009) Character-based DNA barcoding: a superior tool for species classification. *Berliner und Munchener Tierarztliche Wochenschrift* 122: 446-450.

Boero F, Bouillon J (2005) Cnidaria and Ctenophora. *Marine Parasitology*. CSIRO Publishing: 177-182.

Boero F, Bouillon J, Gravili C (2000) A survey of *Zanclea*, *Halocoryne* and *Zanclella* (Cnidaria, Hydrozoa, Anthomedusae, Zancleidae) with description of new species. *Italian Journal of Zoology* 67: 93-124.

Bouillon J, Gravili C, Gili J-M, Boero F (2006) *An Introduction to Hydrozoa*. Publications Scientifiques du Museum, Paris.

Bouillon J, Pagès F, Gili J-M, Palanques A, Puig P, Heussner S (2000) Deep-water Hydromedusae from the Lacaze-Duthiers submarine canyon (Banyuls, northwestern Mediterranean) and description of two new genera, *Guillea* and *Parateclaia*. *Scientia Marina* 64: 87-95.

Budd AF, Fukami H, Smith ND, Knowlton N (2012) Taxonomic classification of the reef coral family Mussidae (Cnidaria: Anthozoa: Scleractinia). *Zoological Journal of the Linnean Society* 166: 465-529.

Calder DR (1988) Shallow-Water Hydroids of Bermuda: The Athecatae. *Royal Ontario Museum Life Sciences Contributions* 148: 1-107.

Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution* 17: 540-552.

Charleston M, Robertson D (2002) Preferential host switching by primate lentiviruses can account for phylogenetic similarity with the primate phylogeny. *Systematic Biology* 51: 528-535.

Churchill CK, Valdés Á, Foighil DÓ (2014) Molecular and morphological systematics of neustonic nudibranchs (Mollusca: Gastropoda: Glaucidae: Glaucus), with descriptions of three new cryptic species. *Invertebrate Systematics* 28: 174-195.

Cruaud A, Rønsted N, Chantarasuwan B, Chou LS, Clement WL, Couloux A et al., Hanson PE (2012) An extreme case of plant-insect codiversification: figs and fig-pollinating wasps. *Systematic Biology* 61: 1029-1047.

Cunningham CW, Buss LW (1993) Molecular evidence for multiple episodes of paedomorphosis in the family Hydractiniidae. *Biochemical Systematics and Ecology* 21: 57-69.

Dayrat B (2005) Towards integrative taxonomy. *Biological Journal of the Linnean Society* 85: 407-415.

DiBattista JD, Howard Choat J, Gaither MR, Hobbs JPA, Lozano-Cortés DF, Myers RF et al., Westneat MW (2016) On the origin of endemic species in the Red Sea. *Journal of Biogeography* 43: 13-30.

Drummond A, Ashton B, Buxton S, Cheung M, Cooper A, Heled J et al., Sturrock S (2010) Geneious v6. 1.6. *Website: http://www. geneious. com*.

Drummond AJ, Suchard MA, Xie D, Rambaut A (2012) Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution* 29: 1969-1973.

Edwards NC, Thomas MB, Long BA, Amyotte SJ (1987) Catecholamines induce metamorphosis in the hydrozoan *Halocordyle disticha* but not in *Hydractinia echinata*. *Development Genes and Evolution* 196: 381-384.

Eshel G, Cane MA, Blumenthal MB (1994) Modes of subsurface, intermediate, and deep water renewal in the Red Sea. *Journal of Geophysical Research: Oceans* 99: 15941-15952.

Excoffier L, Lischer HE (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* 10: 564-567.

Ezard T, Fujisawa T, Barraclough T (2009) Splits: species' limits by threshold statistics. *R package version* 1.

Faucci A, Toonen RJ, Hadfield MG (2007) Host shift and speciation in a coral-feeding nudibranch. *Proceedings of the Royal Society of London B: Biological Sciences* 274: 111-119.

Folino-Rorem NC, Darling JA, D'Ausilio CA (2009) Genetic analysis reveals multiple cryptic invasive species of the hydrozoan genus *Cordylophora*. *Biological Invasions* 11: 1869-1882.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* 3: 294-299.

Fontana S, Keshavmurthy S, Hsieh HJ, Denis V, Kuo C-Y, Hsu C-M et al., Chen CA (2012) Molecular evidence shows low species diversity of coral-associated hydroids in *Acropora* corals. *PloS One* 7: e50130.

Fontaneto D, Flot J-F, Tang CQ (2015) Guidelines for DNA taxonomy, with a focus on the meiofauna. *Marine Biodiversity* 45: 433-451.

Fujisawa T, Barraclough TG (2013) Delimiting species using single-locus data and the Generalized Mixed Yule Coalescent approach: a revised method and evaluation on simulated data sets. *Systematic Biology* 62: 707-724.

Fukami H, Chen CA, Budd AF, Collins A, Wallace C, Chuang Y-Y et al., Sheppard C (2008) Mitochondrial and nuclear genes suggest that stony corals are monophyletic but most families of stony corals are not (Order Scleractinia, Class Anthozoa, Phylum Cnidaria). *PloS One* 3: e3222.

Furby KA, Bouwmeester J, Berumen ML (2013) Susceptibility of central Red Sea corals during a major bleaching event. *Coral Reefs* 32: 505-513.

Gates RD, Ainsworth TD (2011) The nature and taxonomic composition of coral symbiomes as drivers of performance limits in scleractinian corals. *Journal of Experimental Marine Biology and Ecology* 408: 94-101.

Gershwin LA, Zeidler W (2003) Encounter 2002 expedition to the isles of St Francis, South Australia: medusae, siphonophores and ctenophores. *Transactions of the Royal Society of South Australia* 127: 205-241.

Gili J-M, Hughes R (1995) The ecology of marine benthic hydroids. *Oceanography and Marine Biology* 33: 351-426.

Gittenberger A, Reijnen BT, Hoeksema BW (2011) A molecularly based phylogeny reconstruction of mushroom corals (Scleractinia Fungiidae) with taxonomic consequences and evolutionary implications for life history traits. *Contributions to Zoology* 80: 107-132.

Govindarajan AF, Halanych KM, Cunningham CW (2005) Mitochondrial evolution and phylogeography in the hydrozoan *Obelia geniculata* (Cnidaria). *Marine Biology* 146: 213-222.

Gravili C, Boero F, Bouillon J (1996) *Zanclea* species (Hydroidomedusae, Anthomedusae) from the Mediterranean. *Scientia Marina* 60: 99-108.

Guzner B, Novoplansky A, Chadwick N (2007) Population dynamics of the reef-building coral *Acropora hemprichii* as an indicator of reef condition. *Marine Ecology Progress Series* 333: 143-150.

Haeckel EHPA (1879) *Monographie der Medusen*. G. Fischer.

Hirose M, Hirose E (2011) A new species of *Zanclea* (Cnidaria: Hydrozoa) associated with scleractinian corals from Okinawa, Japan. *Journal of the Marine Biological Association of the United Kingdom* 92: 877-884.

Hoeksema BW (2007). Delineation of the Indo-Malayan centre of maximum marine biodiversity: the Coral Triangle. *Biogeography, Time, and Place: Distributions, Barriers, and Islands*. Springer: 117-178.

Hoeksema BW, Van der Meij SET, Fransen CHJM (2012) The mushroom coral as a habitat. *Journal of the Marine Biological Association of the United Kingdom* 92: 647-663.

Huang D, Benzoni F, Arrigoni R, Baird AH, Berumen ML, Bouwmeester J et al., Budd AF (2014a) Towards a phylogenetic classification of reef corals: the Indo-Pacific genera *Merulina, Goniastrea* and *Scapophyllia* (Scleractinia, Merulinidae). *Zoologica Scripta* 43: 531-548.

Huang D, Benzoni F, Fukami H, Knowlton N, Smith ND, Budd AF (2014b) Taxonomic classification of the reef coral families Merulinidae, Montastraeidae, and Diploastraeidae (Cnidaria: Anthozoa: Scleractinia). *Zoological Journal of the Linnean Society* 171: 277-355.

Huang D, Licuanan WY, Hoeksema BW, Chen CA, Ang PO, Huang H et al., Affendi YA (2015) Extraordinary diversity of reef corals in the South China Sea. *Marine Biodiversity* 45: 157-168.

Huelsenbeck JP, Nielsen R, Bollback JP (2003) Stochastic mapping of morphological characters. *Systematic Biology* 52: 131-158.

Hughes TP, Bellwood DR, Connolly SR (2002) Biodiversity hotspots, centres of endemicity, and the conservation of coral reefs. *Ecology Letters* 5: 775-784.

Hughes TP, Graham NA, Jackson JB, Mumby PJ, Steneck RS (2010) Rising to the challenge of sustaining coral reef resilience. *Trends in Ecology & Evolution* 25: 633-642.

Hughes TP, Kerry JT, Álvarez-Noriega M, Álvarez-Romero JG, Anderson KD, Baird AH et al., Berkelmans R (2017) Global warming and recurrent mass bleaching of corals. *Nature* 543: 373-377.

Johnson SB, Warén A, Tunnicliffe V, Dover CV, Wheat CG, Schultz TF, Vrijenhoek RC (2015) Molecular taxonomy and naming of five cryptic species of *Alviniconcha* snails (Gastropoda: Abyssochrysoidea) from hydrothermal vents. *Systematics and Biodiversity* 13: 278-295.

Jörger KM, Schrödl M (2013) How to describe a cryptic species? Practical challenges of molecular taxonomy. *Frontiers in Zoology* 10: 59.

Jörger KM, Schrödl M (2014) How to use CAOS software for taxonomy? A quick guide to extract diagnostic nucleotides or amino acids for species descriptions. *Spixiana* 37: 21-26.

Katoh K, Standley DM (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution* 30: 772-780.

Kitahara MV, Cairns SD, Stolarski J, Blair D, Miller DJ (2010) A comprehensive phylogenetic analysis of the Scleractinia (Cnidaria, Anthozoa) based on mitochondrial CO1 sequence data. *PloS One* 5: e11490.

Kitano YF, Benzoni F, Arrigoni R, Shirayama Y, Wallace CC, Fukami H (2014) A phylogeny of the family Poritidae (cnidaria, scleractinia) based on molecular and morphological analyses. *PloS One* 9: e98406.

Kramp P (1968) The Hydromedusae of the Pacific and Indian Oceans. Sections II and III. *Dana Report* 72: 1-200.

Lanfear R, Calcott B, Ho SY, Guindon S (2012) PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution* 29: 1695-1701.

Leigh JW, Bryant D (2015) popart: full-feature software for haplotype network construction. *Methods in Ecology and Evolution* 6: 1110-1116.

Loya Y, Sakai K, Yamazato K, Nakano Y, Sambali H, van Woesik R (2001) Coral bleaching: the winners and the losers. *Ecology Letters* 4: 122-131.

Maggioni D, Montano S, Seveso D, Galli P (2016) Molecular evidence for cryptic species in *Pteroclava krempfi* (Hydrozoa, Cladocorynidae) living in association with alcyonaceans. *Systematics and Biodiversity* 14: 484-493.

Marcum BA, Diehl FA (1978) Anchoring cells (desmocytes) in the hydrozoan polyp *Cordylophora*. *Tissue and Cell* 10: 113-124.

Marshall P, Baird A (2000) Bleaching of corals on the Great Barrier Reef: differential susceptibilities among taxa. *Coral Reefs* 19: 155-163.

Medlin L, Elwood HJ, Stickel S, Sogin ML (1988) The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene* 71: 491-499.

Miglietta MP, Lessios HA (2009) A silent invasion. *Biological Invasions* 11: 825-834.

Miglietta MP, Schuchert P, Cunningham CW (2009) Reconciling genealogical and morphological species in a worldwide study of the Family Hydractiniidae (Cnidaria, Hydrozoa). *Zoologica Scripta* 38: 403-430.

Millard NAH (1975) Monograph on the Hydroida of Southern Africa. *Annales of the South African Museum* 68: 1-513.

Millard NAH, Bouillon J (1974) A collection of hydroids from Moçambique, East Africa. *Annales of the South African Museum* 65: 1-40.

Monaghan MT, Wild R, Elliot M, Fujisawa T, Balke M, Inward DJ et al., Vogler AP (2009) Accelerated species inventory on Madagascar using coalescent-based models of species delineation. *Systematic Biology* 58: 298-311.

Montano S, Arrigoni R, Pica D, Maggioni D, Puce S (2015a) New insights into the symbiosis between *Zanclea* (Cnidaria, Hydrozoa) and scleractinians. *Zoologica Scripta* 44: 92-105.

Montano S, Galli P, Maggioni D, Seveso D, Puce S (2014) First record of coral-associated *Zanclea* (Hydrozoa, Zancleidae) from the Red Sea. *Marine Biodiversity* 44: 581-584.

Montano S, Maggioni D, Galli P, Seveso D, Puce S (2013) *Zanclea*-coral association new records from Maldives. *Coral Reefs* 32: 701.

Montano S, Seveso D, Galli P, Obura DO (2010) Assessing coral bleaching and recovery with a colour reference card in Watamu Marine Park, Kenya. *Hydrobiologia* 655: 99-108.

Montano S, Seveso D, Galli P, Puce S, Hoeksema BW (2015b) Mushroom corals as hosts of the hydrozoan symbiont *Zanclea* sp. *Marine Biology Research* 11: 773-779.

Morard R, Escarguel G, Weiner AK, André A, Douady CJ, Wade CM et al., Quillévéré F (2016) Nomenclature for the nameless: a proposal for an integrative molecular taxonomy of cryptic diversity exemplified by planktonic foraminifera. *Systematic Biology* 65: 925-940.

Moura CJ, Cunha MR, Porteiro FM, Rogers AD (2011) The use of the DNA barcode gene 16S mRNA for the clarification of taxonomic problems within the family Sertulariidae (Cnidaria, Hydrozoa). *Zoologica Scripta* 40: 520-537.

Moura CJ, Cunha MR, Porteiro FM, Rogers AD (2012a) Polyphyly and cryptic diversity in the hydrozoan families Lafoeidae and Hebellidae (Cnidaria: Hydrozoa). *Invertebrate Systematics* 25: 454-470.

Moura CJ, Cunha MR, Porteiro FM, Yesson C, Rogers AD (2012b) Evolution of *Nemertesia* hydroids (Cnidaria: Hydrozoa, Plumulariidae) from the shallow and deep waters of the NE Atlantic and western Mediterranean. *Zoologica Scripta* 41: 79-96.

Moura CJ, Harris DJ, Cunha MR, Rogers AD (2008) DNA barcoding reveals cryptic diversity in marine hydroids (Cnidaria, Hydrozoa) from coastal and deep-sea environments. *Zoologica Scripta* 37: 93-108.

Müller W (1969) Auslösung der Metamorphose durch Bakterien bei den Larven von *Hydractinia echinata*. *Zoologische Jahrbücher* 86: 84-95.

Müller WA, Leitz T (2002) Metamorphosis in the Cnidaria. *Canadian Journal of Zoology* 80: 1755-1771.

Munday PL, van Herwerden L, Dudgeon CL (2004) Evidence for sympatric speciation by host shift in the sea. *Current Biology* 14: 1498-1504.

Muscatine L, Tambutte E, Allemand D (1997) Morphology of coral desmocytes, cells that anchor the calicoblastic epithelium to the skeleton. *Coral Reefs* 16: 205-213.

Osman RW, Haugsness JA (1981) Mutualism among sessile invertebrates: a mediator of competition and predation. Science 211: 846-848.

Pantos O, Bythell JC (2010) A novel reef coral symbiosis. *Coral Reefs* 29: 761-770.

Pantos O, Hoegh-Guldberg O (2011) Shared skeletal support in a coral-hydroid symbiosis. *PloS One* 6: e20946.

Paradis E, Strimmer K, Claude J, Jobb G, Opgen-Rhein R, Dutheil J et al., Lemon J (2004) ape: Analyses of Phylogenetics and Evolution. R package version 1.3-1. *Comprehensive R Archive Network, Vienna, Austria*.

Pearson R (1981) Recovery and recolonization of coral reefs. *Marine Ecology Progress Series* 4: 105-122.

Peña-Cantero ÁL, Sentandreu V (2017) Phylogenetic relationships of endemic Antarctic species of *Staurotheca* Allman, 1888 (Cnidaria, Hydrozoa). *Polar Biology* 40: 301-312.

Pica D, Bastari A, Vaga CF, Di Camillo CG, Montano S, Puce S (2017) Hydroid diversity of Eilat Bay with the description of a new *Zanclea* species. *Marine Biology Research* 13: 467-479.

Pons J, Barraclough TG, Gomez-Zurita J, Cardoso A, Duran DP, Hazell S et al., Vogler AP (2006) Sequence-based species delimitation for the DNA taxonomy of undescribed insects. *Systematic Biology* 55: 595-609.

Puce S, Bavestrello G, Di Camillo CG, Boero F (2007) Symbiotic relationships between hydroids and bryozoans. *Symbiosis* 44: 137-143.

Puce S, Cerrano C, Boyer M, Ferretti C, Bavestrello G (2002) *Zanclea* (Cnidaria: Hydrozoa) species from Bunaken Marine Park (Sulawesi Sea, Indonesia). *Journal of the Marine Biological Association of the United Kingdom* 82: 943-954.

Puce S, Cerrano C, Di Camillo CG, Bavestrello G (2008a) Hydroidomedusae (Cnidaria: Hydrozoa) symbiotic radiation. *Journal of the Marine Biological Association of the United Kingdom* 88: 1715-1721.

Puce S, Di Camillo CG, Bavestrello G (2008b) Hydroids symbiotic with octocorals from the Sulawesi Sea, Indonesia. *Journal of the Marine Biological Association of the United Kingdom* 88: 1643-1654.

Puillandre N, Lambert A, Brouillet S, Achaz G (2012) ABGD, Automatic Barcode Gap Discovery for primary species delimitation. *Molecular Ecology* 21: 1864-1877.

Raitsos DE, Pradhan Y, Brewin RJ, Stenchikov G, Hoteit I (2013) Remote sensing the phytoplankton seasonal succession of the Red Sea. *PloS One* 8: e64909.

Rambaut A, Drummond A (2013) TreeAnnotator v1. 7.0. *Available as part of the BEAST package at http://beast. bio. ed. ac. uk*.

Rambaut A, Suchard M, Xie W, Drummond A (2014) Tracer v. 1.6. Institute of Evolutionary Biology, University of Edinburgh.

Reid NM, Carstens BC (2012) Phylogenetic estimation error can decrease the accuracy of species delimitation: a Bayesian implementation of the general mixed Yule-coalescent model. *BMC Evolutionary Biology* 12: 196.

Revell LJ (2012) phytools: an R package for phylogenetic comparative biology (and other things). *Methods in Ecology and Evolution* 3: 217-223.

Revell LJ (2013) Two new graphical methods for mapping trait evolution on phylogenies. *Methods in Ecology and Evolution* 4: 754-759.

Ristedt H, Schuhmacher H (1985) The bryozoan *Rhynchozoon larreyi* (Audouin, 1826)-A successful competitor in coral reef communities of the Red Sea. *Marine Ecology* 6: 167-179.

Romano SL, Palumbi SR (1996) Evolution of scleractinian corals inferred from molecular systematics. *Science* 271: 640-642.

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S et al., Huelsenbeck JP (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* 61: 539-542.

Sammarco PP, Carleton JJ (1981). Damselfish territoriality and coral community structure: reduced grazing, coral recruitment, and effects on coral spat. *Proceedings of the 4th International Coral Reef Symposium* 2: 525-535.

Sarkar IN, Planet PJ, Desalle R (2008) CAOS software for use in character-based DNA barcoding. *Molecular Ecology Resources* 8: 1256-1259.

Sarkar IN, Thornton JW, Planet PJ, Figurski DH, Schierwater B, DeSalle R (2002) An automated phylogenetic key for classifying homeoboxes. *Molecular Phylogenetics and Evolution* 24: 388-399.

Scarpa F, Cossu P, Lai T, Sanna D, Curini-Galletti M, Casu M (2016) Meiofaunal cryptic species challenge species delimitation: the case of the *Monocelis lineata* (Platyhelminthes: Proseriata) species complex. *Contributions to Zoology* 85: 123-145.

Schuchert P (2014) High genetic diversity in the hydroid *Plumularia setacea*: a multitude of cryptic species or extensive population subdivision? *Molecular Phylogenetics and Evolution* 76: 1-9.

Shipman C, Gosliner T (2015) Molecular and morphological systematics of *Doto* Oken, 1851 (Gastropoda: Heterobranchia), with descriptions of five new species and a new genus. *Zootaxa* 3973: 57-101.

Sofianos SS, Johns WE (2003) An oceanic general circulation model (OGCM) investigation of the Red Sea circulation: 2. Three-dimensional circulation in the Red Sea. *Journal of Geophysical Research: Oceans* 108.

Stabili L, Gravili C, Pizzolante G, Lezzi M, Tredici SM, De Stefano M, Boero F, Alifano P (2017). *Aglaophenia octodonta* (Cnidaria, Hydrozoa) and the associated microbial community: a cooperative alliance? *Microbial ecology*: 1-14.

Stella JS, Pratchett MS, Hutchings PA, Jones GP (2011) Coral-associated invertebrates: diversity, ecological importance and vulnerability to disturbance. *Oceanography and Marine Biology: an Annual Review* 49: 43-104.

Stepanjants S (1972) Hydroidea of the coastal waters of the Davis Sea (collected by the XIth Soviet Antarctic Expedition of 1965-1966). *Biological Reports of the Sovietic Antarctic Expedition* 5: 56-79.

Sukumaran J, Holder MT (2010) DendroPy: a Python library for phylogenetic computing. *Bioinformatics* 26: 1569-1571.

Talavera G, Castresana J (2007) Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Systematic Biology* 56: 564-577.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution* 30: 2725-2729.

Terraneo TI, Arrigoni R, Benzoni F, Tietbohl MD, Berumen ML (2017) Exploring the genetic diversity of shallow-water Agariciidae (Cnidaria: Anthozoa) from the Saudi Arabian Red Sea. *Marine Biodiversity*: 1-14.

Thomas MB, Freeman G, Martin VJ (1987) The embryonic origin of neurosensory cells and the role of nerve cells in metamorphosis in *Phialidium gregarium* (Cnidaria, Hydrozoa). *International Journal of Invertebrate Reproduction and Development* 11: 265-285.

Tsang LM, Chan BKK, Shih FL, Chu KH, Chen CA (2009) Host-associated speciation in the coral barnacle *Wanella milleporae* (Cirripedia: Pyrgomatidae) inhabiting the Millepora coral. *Molecular Ecology* 18: 1463-1475.

Uchida T, Sugiura Y (1976) On a Hydromedusa, *Zanclea prolifera* n. sp., of which the medusa gives rise to medusa-buds. *Proceedings of the Japan Academy* 52: 141-144.

Veron J (2000) *Corals of the World*. Australian Institute of Marine Science.

Wallace CC (1985) Reproduction, recruitment and fragmentation in nine sympatric species of the coral genus *Acropora*. *Marine Biology* 88: 217-233.

Wallace CC, Chen CA, Fukami H, Muir PR (2007) Recognition of separate genera within *Acropora* based on new morphological, reproductive and genetic evidence from *Acropora togianensis*, and elevation of the subgenus *Isopora* Studer, 1878 to genus (Scleractinia: Astrocoeniidae; Acroporidae). *Coral Reefs* 26: 231-239.

Xu Z, Huang J, Chen X (1991) On new species and record of Hydromedusae in the upwelling region off the Minnan-Taiwan Bank fishing ground, China. *Minnan-Taiwan Bank Fishing Ground Upwelling Ecosystem Study*. Beijing Science Press: 469-486.

Xu Z, Huang J, Guo D (2008). Six new species of Anthomedusae (Hydrozoa, Hydroidomedusae) from Beibu Gulf, China. *Symposium on Oceanography of the Beibu Gulf*. China Ocean Press.

Zhang J, Kapli P, Pavlidis P, Stamatakis A (2013) A general species delimitation method with applications to phylogenetic placements. *Bioinformatics* 29: 2869-2876.

Zheng L, He J, Lin Y, Cao W, Zhang W (2014) 16S rRNA is a better choice than COI for DNA barcoding hydrozoans in the coastal waters of China. *Acta Oceanologica Sinica* 33: 55-76.

Zielske S, Haase M (2015) Molecular phylogeny and a modified approach of character-based barcoding refining the taxonomy of New Caledonian freshwater gastropods (Caenogastropoda, Truncatelloidea, Tateidae). *Molecular Phylogenetics and Evolution* 89: 171-181.

Zietara MS, Arndt A, Geets A, Hellemans B, Volckaert FA (2000) The nuclear rDNA region of *Gyrodactylus arcuatus* and *G. branchicus* (Monogenea: Gyrodactylidae). *Journal of Parasitology* 86: 1368-1373.

Zou S, Li Q, Kong L, Yu H, Zheng X (2011) Comparing the usefulness of distance, monophyly and character-based DNA barcoding methods in species identification: a case study of Neogastropoda. *PloS One* 6: e26619.

Zwickl D (2006) GARLI: genetic algorithm for rapid likelihood inference. *See http://www. http://www.bio.utexas.edu/faculty/antisense/garli/Garli.html*.

2.7. TABLES

Table 2.1. Genetic distances (uncorrected p-distances in %) of the A) *16S*, B) *COX1*, C) *COX3* sequences among and within the clades recovered with the phylogenetic analyses. Values are indicated as mean ± standard deviation.

A) *16S*	I	II	III	IVa	VI	IVb (*Z. gallii*)	V (*Z. sango*)
Clade I	0 ± 0						
Clade II	3.5 ± 0.9	0.3 ± 0.2					
Clade III	4.3 ± 1	3.3 ± 0.9	0.1 ± 0.1				
Clade IVa	5.9 ± 1.2	5.5 ± 1.1	3.8 ± 1	0.2 ± 0.1			
Clade VI	5.1 ± 1.2	5.3 ± 1.1	4.9 ± 1.1	4.1 ± 1	0.4 ± 0.1		
Z. gallii (IVb)	5.6 ± 1.2	6 ± 1.2	4.8 ± 1.1	3.8 ± 1	4.9 ± 1.1	0.7 ± 0.3	
Z. sango (V)	4.5 ± 1	5.2 ± 1	5.4 ± 1.1	5.1 ± 1.1	3.2 ± 0.8	5.9 ± 1.2	1.1 ± 0.3

B) *COX1*	I	II	III	IVa	VI	IVb (*Z. gallii*)	V (*Z. sango*)
Clade I	0.3 ± 0.1						
Clade II	4.8 ± 0.8	0.7 ± 0.2					
Clade III	6.7 ± 0.9	4.9 ± 0.8	0.2 ± 0.1				
Clade IVa	9.1 ± 1	8.5 ± 1	8.1 ± 1	0.8 ± 0.2			
Clade VI	7.6 ± 1	7.5 ± 1	8.1 ± 1	9.1 ± 1	0.5 ± 0.1		
Z. gallii (IVb)	8.6 ± 1	7.8 ± 1	7 ± 0.9	6.8 ± 0.9	8.7 ± 1	0.9 ± 0.2	
Z. sango (V)	8 ± 1	7.1 ± 0.9	7.5 ± 0.9	8.2 ± 1	5 ± 0.8	7.6 ± 0.9	1.2 ± 0.3

C) *COX3*	I	II	III	IVa	VI	IVb (*Z. gallii*)	V (*Z. sango*)
Clade I	0.1 ± 0.1						
Clade II	9.3 ± 1.1	1 ± 0.3					
Clade III	8.8 ± 1.1	8.2 ± 1	0.6 ± 0.2				
Clade IVa	9.6 ± 1.1	10.5 ± 1.2	9.7 ± 1.1	1 ± 0.3			
Clade VI	10.8 ± 1.2	10.8 ± 1.2	10.4 ± 1.1	9.5 ± 1.1	0.4 ± 0.1		
Z. gallii (IVb)	8.7 ± 1.1	11 ± 1.1	9.6 ± 1.1	8.5 ± 1	10.4 ± 1.2	1.6 ± 0.3	
Z. sango (V)	11.3 ± 1.2	10.9 ± 1.2	10.5 ± 1.1	9.5 ± 1.1	5.3 ± 0.8	10.7 ± 1.2	1 ± 0.3

Table 2.2. Summary of species delimitation outputs. The presence of a lineage (+) or a further subdivision in more lineages (f.s.) is indicated for ABGD (JC, K2P, p-dist), PTP (PTP, bPTP), stGMYC, mtGMYC and bGMYC methods. *nomenclature after Morard et al. (2016)

Clade	Species name*	Species delimitation				
		ABGD	PTP/bPTP	stGMYC	mtGMYC	bGMYC
I	*Zanclea intermedia* I	+	+	+	+	+
II	*Zanclea intermedia* II	+	f.s.	f.s.	+	f.s.
III	*Zanclea intermedia* III	+	+	+	+	+
IVa	*Zanclea gallii* II	+	+	+	+	+
IVb	*Zanclea gallii* I	+	f.s.	+	+	f.s.
V	*Zanclea sango* I	f.s.	+	+	+	+
VI	*Zanclea sango* II	+	+	f.s.	f.s	f.s.

Table 2.3. Diagnostic molecular characters in the A) *16S*, B) *COX1*, C) *COX3* sequences for the recovered species

	Diagnostic characters with position in alignment (in reference sequence)
A) *16S* (LT607008)	
Z. intermedia I	141 (277), G; 206 (342), C
Z. intermedia II	156 (292), C; 195 (331), G; 300 (436), G; 311 (447), C
Z. intermedia III	36 (172), G; 161 (297), G
Z. gallii I	139 (275), T; 145 (281), G; 161 (297), C; 326 (462), G
Z. gallii II	161 (297), T; 223 (359), G; 233 (369), G
Z. sango I	76 (212), G; 207 (343), C; 256 (392), G
Z. sango II	140 (276), G; 162 (298), C; 253 (389), C; 313 (449), C
B) *COX1* (LT607016)	
Z. intermedia I	75 (105), T; 127 (157), C; 236 (266), C; 276 (306), G; 306 (336), G; 345 (375), G; 489 (519), C
Z. intermedia II	69 (99), G; 268 (298), C; 510 (540), G; 582 (612), T; 585 (685), C
Z. intermedia III	247 (277), C; 399 (429), C; 408 (438), T; 417 (447); 565 (595), C; 615 (645), C
Z. gallii I	84 (114), G; 549 (579), G; 579 (609), C; 588 (618), C; 612 (642), G; 615 (645), A
Z. gallii II	18 (48), G; 24 (54), G; 36 (66), G; 141 (171), C; 156 (186), G; 240 (270), C; 306 (336), T; 372 (402), G; 387 (417), G; 501 (531), C; 570 (600), C; 633 (663), G
Z. sango I	93 (123), C; 255 (285), G; 531 (561), C
Z. sango II	33 (63),C; 138 (168), C; 228 (258), G; 239 (269), T; 378 (408), C; 381 (411), C
C) *COX3* (KT824445)	
Z. intermedia I	66 (64), C; 99 (97), G; 150 (148), C; 165 (163), C; 195 (193), C; 279 (277), C; 420 (418), G; 438 (436), G; 588 (586), G; 607 (605), C
Z. intermedia II	126 (124), G; 228 (226), G; 273 (271), C; 348 (346), A; 393 (391), C; 414 (412), G; 454 (452), G; 555 (553), T
Z. intermedia III	108 (106), C; 216 (214), G; 219 (217), C; 234 (234), T; 280 (278), A; 363 (361), G; 467 (465), C; 483 (481), G; 516 (514), C
Z. gallii I	243 (241), C; 297 (295), C; 307 (305), A; 316 (314), A; 318 (316), C; 345 (343), G; 360 (358), C; 372 (370), G; 419 (417), G; 422 (420), A
Z. gallii II	63 (61), C; 159 (157), T; 300 (298), G; 322 (320), C; 372 (370), T; 408 (406), G; 510 (508), C; 573 (571), C; 610 (608), C; 615 (613), G
Z. sango I	9 (7), G; 117 (115), C; 124 (122), C; 132 (130), C; 411 (409), C; 549 (547), G; 564 (562), C; 609 (607), G
Z. sango II	33 (31), G; 76 (74), C; 205 (203), C; 237 (235), C; 309 (307), C; 540 (538), G

Table 2.4. Molecular diversity indices and neutrality tests for A) *16S*, B) *COX1*, C) *COX3*, showing number of individuals (N), number of haplotypes (H), gene diversity (h), average nucleotide diversity (π), Tajima's D and Fu's Fs. Significant values in bold ($p < 0.05$).

A) *16S*	Molecular diversity indices				Neutrality	
	N	H	h	π	Tajima's D	Fu's Fs
***Zanclea intermedia* I**	20	1	0	0		
Indo-Pacific	11	1	0	0	0	n. c.
Red Sea	9	1	0	0	0	n. c.
***Zanclea intermedia* II**	10	4	0.644 ± 0.152	0.003 ± 0.003		
Indo-Pacific	6	1	0	0	0	n. c.
Red Sea	4	3	0.833 ± 0.222	0.004 ± 0.004	-0.754	-0.288
***Zanclea intermedia* III**	15	1	0	0		
Indo-Pacific	8	1	0	0	0	n. c.
Red Sea	7	1	0	0	0	n. c.
***Zanclea gallii* I**	34	8	0.779 ± 0.048	0.007 ± 0.004		
Indo-Pacific	32	7	0.752 ± 0.051	0.006 ± 0.004	-0.001	0.001
Red Sea	2	1	0	0	0	n. c.
***Zanclea gallii* II**	6	3	0.67 ± 0.215	0.003 ± 0.003		
Red Sea	6	3	0.6 ± 0.215	0.003 ± 0.003	-0.447	0.117
***Zanclea sango* I**	14	3	0.56 ± 0.124	0.011 ± 0.007		
Indo-Pacific	12	3	0.621 ± 0.118	0.012 ± 0.007	1.326	5.014
Red Sea	2	1	0	0	0	n. c.
***Zanclea sango* II**	121	14	0.589± 0.046	0.003 ± 0.002		
Indo-Pacific	74	3	0.177 ± 0.058	0.001 ± 0.001	-0.98	0.306
Red Sea	45	13	0.719 ± 0.067	0.005 ± 0.003	**-1.762**	**-6.099**
Caribbean Sea	2	1	0	0	0	n. c.

B) *COX1*	Molecular diversity indices				Neutrality	
	N	H	h	π	Tajima's D	Fu's Fs
***Zanclea intermedia* I**	20	5	0.7 ± 0.066	0.003 ± 0.002		
Indo-Pacific	11	4	0.6 ± 0.154	0.004± 0.002	-1.264	1.203
Red Sea	9	2	0.222 ± 0.166	0.001 ± 0.001	-1.088	-0.263
***Zanclea intermedia* II**	9	4	0.694 ± 0.147	0.007 ± 0.005		
Indo-Pacific	5	1	0	0	0	n. c.
Red Sea	4	3	0.833 ± 0.222	0.005 ± 0.004	-0.314	0.811
***Zanclea intermedia* III**	13	4	0.68 ± 0.089	0.002 ± 0.001		
Indo-Pacific	6	2	0.333 ± 0.215	0.001 ± 0.001	-0.933	-0.003
Red Sea	7	2	0.286 ± 0.196	0.001 ± 0.001	-1.006	-0.095
***Zanclea gallii* I**	10	4	0.822 ± 0.072	0.009 ± 0.005		
Indo-Pacific	8	3	0.75 ± 0.97	0.009 ± 0.006	0.888	4.569
Red Sea	2	1	0	0	0	n. c.
***Zanclea gallii* II**	6	4	0.8 ± 0.172	0.007 ± 0.005		
Red Sea	6	4	0.8 ± 0.172	0.007 ± 0.005	0.302	1.021
***Zanclea sango* I**	10	3	0.689 ± 0.104	0.011 ± 0.007		
Indo-Pacific	8	2	0.536 ± 0.123	0.012 ± 0.007	1.991	8.791
Red Sea	2	1	0	0	0	n. c.
***Zanclea sango* II**	118	18	0.562 ± 0.053	0.005 ± 0.003		
Indo-Pacific	70	6	0.413 ± 0.07	0.003 ± 0.002	-1.229	1.738
Red Sea	46	15	0.721 ± 0.071	0.006± 0.004	**-2.135**	-2.631
Caribbean Sea	2	1	0	0	0	n. c.

C) *COX3*	Molecular diversity indices				Neutrality	
	N	H	h	π	Tajima's D	Fu's Fs
***Zanclea intermedia* I**	20	2	0.505 ± 0.056	0.002 ± 0.002		
Indo-Pacific	11	1	0	0	0	n. c.
Red Sea	9	2	0.222 ± 0.166	0.001 ± 0.001	-1.513	1.318
***Zanclea intermedia* II**	9	3	0.639 ± 0.126	0.01 ± 0.006		
Indo-Pacific	5	1	0	0	0	n. c.
Red Sea	4	2	0.5 ± 0.265	0.009 ± 0.006	-0.837	4.22
***Zanclea intermedia* III**	14	4	0.714 ± 0.079	0.002 ± 0.001		
Indo-Pacific	8	2	0.429± 0.169	0.001 ± 0.001	0.414	1.653
Red Sea	6	2	0.333 ± 0.215	0.001 ± 0.001	-0.933	-0.003
***Zanclea gallii* I**	8	5	0.857 ± 0.108	0.016 ± 0.009		
Indo-Pacific	5	2	0.6 ± 0.175	0.006 ± 0.004	1.718	3.967
Red Sea	3	3	1 ± 0.272	0.016 ± 0.013	0	1.066
***Zanclea gallii* II**	6	6	1 ± 0.096	0.009 ± 0.006		
Red Sea	6	6	1 ± 0.096	0.009 ± 0.006	-0.171	-1.964
***Zanclea sango* I**	8	2	0.536 ± 0.123	0.009 ± 0.006		
Indo-Pacific	6	2	0.6 ± 0.129	0.01 ± 0.007	2.238	6.464
Red Sea	2	1	0	0	0	n. c.
***Zanclea sango* II**	115	11	0.356 ± 0.057	0.005 ± 0.003		
Indo-Pacific	67	4	0.247 ± 0.067	0.003 ± 0.002	-0.808	4.02
Red Sea	46	9	0.453 ± 0.091	0.006 ± 0.003	**-1.644**	0.929
Caribbean Sea	2	1	0	0	0	n. c.

Table 2.5. Analyses of molecular variance (AMOVA) for coral-associated *Zanclea* species and populations inferred from the A) *16S*, B) *COX1*, C) *COX3* sequences. Significant values in bold ($p < 0.05$).

A) *16S*					
source of variation	d.f.	sum of squares	variance components	% of variation	fixation indices
all species					
among species	6	1176.812	7.984	90.39	Fct = **0.326**
among populations within species	7	28.408	0.276	3.13	Fsc = **0.935**
within populations	206	117.91	0.572	6.48	Fst = **0.904**
total	219	1323.13	8.832	100	
***Zanclea intermedia* I**					
among populations within species	1	0	0	0	
within populations	18	0	0	0	Fst = 0
total	19	0	0	0	
***Zanclea intermedia* II**					
among populations within species	1	2.865	0.538	65.57	
within populations	8	2.26	0.283	34.43	Fst = **0.656**
total	9	5.125	0.821	100	
***Zanclea intermedia* III**					
among populations within species	1	0	0	0	
within populations	13	0	0	0	Fst = 0
total	14	0	0	0	
***Zanclea gallii* I**					
among populations within species	1	7.68	1.751	61.62	
within populations	32	34.883	1.09	38.38	Fst = **0.616**
total	33	42.563	2.841	100	
***Zanclea sango* I**					
among populations within species	1	1.887	-0.047	-2.35	
within populations	12	24.578	2.048	102.35	Fst = -0.235
total	13	26.466	2.001		
***Zanclea sango* II**					
among populations within species	2	15.975	0.255	35.58	
within populations	118	54.518	0.462	64.42	Fst = **0.356**
total	120	70.493	0.717		

B) *COX1*

source of variation	d.f.	sum of squares	variance components	% of variation	fixation indices
all species					
among species	6	2389.73	22.07	91.8	Fct = **0.284**
among populations within species	7	57.913	0.56	2.33	Fsc = **0.941**
within populations	172	242.561	1.41	5.87	Fst = **0.918**
total	185	2690.204	24.04		
***Zanclea intermedia* I**					
among populations within species	1	4.811	0.414	36.62	
within populations	18	12.889	0.716	63.68	Fst = **0.366**
total	19	17.7	1.13		
***Zanclea intermedia* II**					
among populations within species	1	14.583	3.123	82.18	
within populations	7	4.75	0.679	17.82	Fst = **0.822**
total	8	19.333	3.802		
***Zanclea intermedia* III**					
among populations within species	1	5.694	0.857	84.8	
within populations	11	1.69	0.154	15.2	Fst = **0.848**
total	12	7.385	1.011		
***Zanclea gallii* I**					
among populations within species	1	6.475	1.218	32.08	
within populations	8	20.625	2.578	67.92	Fst = 0.321
total	9	27.1	3.796		
***Zanclea sango* I**					
among populations within species	1	4.975	0.456	11.48	
within populations	8	28.125	3.517	88.52	Fst = 0.115
total	9	33.1	3.973		
***Zanclea sango* II**					
among populations within species	2	21.375	3.317	18.27	
within populations	115	162.981	1.417	81.73	Fst = **0.183**
total	117	184.356	4.734		

C) *COX3*

source of variation	d.f.	sum of squares	variance components	% of variation	fixation indices
all species					
among species	6	3066.359	29.671	94.23	Fct = **0.942**
among populations within species	7	65.788	0.676	2.15	Fsc = **0.372**
within populations	166	189.275	1.14	3.62	Fst = **0.964**
total	179	3321.422	31.487		
***Zanclea intermedia* I**					
among populations within species	1	3.918	0.391	88.76	
within populations	18	0.89	0.05	11.24	Fst = **0.888**
total	19	4.808	0.441		
***Zanclea intermedia* II**					
among populations within species	1	16.235	3.383	73.83	
within populations	7	8.394	1.199	26.17	Fst = **0.738**
total	8	24.629	4.582		
***Zanclea intermedia* III**					
among populations within species	1	2.276	0.285	47.09	
within populations	12	3.845	0.32	52.91	Fst = **0.471**
total	13	6.121	0.605		
***Zanclea gallii* I**					
among populations within species	1	17.256	3.828	56.89	
within populations	6	17.403	2.9	43.11	Fst = **0.569**
total	7	34.658	6.728		
***Zanclea sango* I**					
among populations within species	1	4.204	0.467	14.29	
within populations	6	16.814	2.802	85.71	Fst = 0.143
total	7	21.018	3.269		
***Zanclea sango* II**					
among populations within species	2	21.901	0.341	23.01	
within populations	112	127.769	1.141	76.99	Fst = **0.23**
total	114	149.67	1.482		

Table 2.6. Pairwise *Fst* values for coral-associated *Zanclea* species and populations inferred from A) *16S*, B) *COX1*, C) *COX3* datasets. Both values for pairwise *Fst* and for correspondent associated p-values are presented below and above the main diagonal, respectively. Significant *Fst* values in bold ($p < 0.05$). IP: Indo-Pacific, RS: Red Sea, CS: Caribbean Sea. Intra-specific comparisons coloured following Figure 2.6.

A) *16S*			1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	*Z. intermedia* I	IP		0.999	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.022	0.000	0.022	0.000	0.010
2	*Z. intermedia* I	RS	0.000		0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.015	0.000	0.012	0.000	0.019
3	*Z. intermedia* II	IP	**1.000**	**1.000**		0.005	0.000	0.000	0.002	0.000	0.000	0.036	0.000	0.041	0.000	0.042
4	*Z. intermedia* II	RS	**0.974**	**0.970**	**0.656**		0.000	0.000	0.005	0.000	0.000	0.087	0.000	0.078	0.000	0.061
5	*Z. intermedia* III	IP	**1.000**	**1.000**	**1.000**	**0.963**		0.999	0.000	0.000	0.000	0.021	0.000	0.021	0.000	0.014
6	*Z. intermedia* III	RS	**1.000**	**1.000**	**1.000**	**0.959**	0.000		0.001	0.000	0.000	0.019	0.000	0.030	0.000	0.041
7	*Z, gallii* II	RS	**0.989**	**0.988**	**0.983**	**0.952**	**0.979**	**0.977**		0.000	0.000	0.033	0.000	0.030	0.000	0.034
8	*Z. sango* II	IP	**0.979**	**0.978**	**0.977**	**0.975**	**0.977**	**0.977**	**0.968**		0.000	0.003	0.000	0.001	0.000	0.002
9	*Z. sango* II	RS	**0.926**	**0.924**	**0.919**	**0.916**	**0.919**	**0.917**	**0.895**	**0.280**		0.003	0.000	0.001	0.000	0.006
10	*Z. sango* II	CS	**1.000**	**1.000**	**1.000**	0.940	**1.000**	**1.000**	**0.964**	**0.859**	**0.597**		0.001	0.311	0.007	0.350
11	*Z. gallii* I	IP	**0.918**	**0.914**	**0.911**	**0.905**	**0.897**	**0.895**	**0.860**	**0.942**	**0.892**	**0.884**		0.002	0.000	0.003
12	*Z. gallii* I	RS	**1.000**	**1.000**	**1.000**	0.943	**1.000**	**1.000**	**0.951**	**0.970**	**0.890**	1.000	**0.615**		0.008	0.323
13	*Z. sango* I	IP	**0.857**	**0.844**	**0.838**	**0.814**	**0.861**	**0.854**	**0.827**	**0.901**	**0.798**	**0.722**	**0.864**	**0.788**		0.499
14	*Z. sango* I	RS	**1.000**	**1.000**	**1.000**	0.946	**1.000**	**1.000**	**0.971**	**0.965**	**0.874**	1.000	**0.908**	1.000	-0.024	

B) *COX1*			1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	*Z. intermedia* I	IP		0.000	0.000	0.000	0.001	0.000	0.001	0.000	0.000	0.015	0.000	0.015	0.000	0.011
2	*Z. intermedia* I	RS	**0.366**		0.000	0.002	0.001	0.000	0.000	0.000	0.000	0.022	0.000	0.010	0.001	0.020
3	*Z. intermedia* II	IP	**0.944**	**0.995**		0.009	0.001	0.004	0.002	0.000	0.000	0.031	0.000	0.053	0.000	0.039
4	*Z. intermedia* II	RS	**0.922**	**0.969**	**0.823**		0.002	0.003	0.006	0.000	0.000	0.057	0.001	0.082	0.000	0.057
5	*Z. intermedia* III	IP	**0.962**	**0.994**	**0.994**	**0.957**		0.000	0.006	0.000	0.000	0.032	0.000	0.026	0.000	0.048
6	*Z. intermedia* III	RS	**0.964**	**0.995**	**0.995**	**0.961**	**0.848**		0.003	0.000	0.000	0.040	0.000	0.037	0.000	0.037
7	*Z. gallii* II	RS	**0.949**	**0.970**	**0.955**	**0.932**	**0.956**	**0.958**		0.000	0.000	0.021	0.000	0.033	0.000	0.044
8	*Z. sango* II	IP	**0.958**	**0.964**	**0.963**	**0.958**	**0.965**	**0.966**	**0.964**		0.479	0.002	0.000	0.002	0.000	0.000
9	*Z. sango* II	RS	**0.925**	**0.934**	**0.929**	**0.921**	**0.935**	**0.936**	**0.934**	-0.005		0.014	0.000	0.001	0.000	0.000
10	*Z. sango* II	CS	**0.958**	**0.996**	**1.000**	0.952	**0.995**	**0.996**	**0.942**	**0.833**	**0.690**		0.019	0.324	0.028	0.329
11	*Z. gallii* I	IP	**0.934**	**0.952**	**0.930**	**0.906**	**0.925**	**0.930**	**0.885**	**0.959**	**0.927**	**0.916**		0.047	0.001	0.017
12	*Z. gallii* I	RS	**0.965**	**0.997**	1.000	0.957	**0.994**	**0.995**	**0.920**	**0.966**	**0.934**	1.000	**0.319**		0.014	0.324
13	*Z. sango* I	IP	**0.909**	**0.928**	**0.895**	**0.866**	**0.907**	**0.914**	**0.882**	**0.917**	**0.858**	**0.798**	**0.866**	**0.869**		0.105
14	*Z. sango* I	RS	**0.961**	**0.997**	**1.000**	0.950	**0.994**	**0.995**	**0.932**	**0.940**	**0.885**	1.000	**0.900**	1.000	0.114	

C) *COX3*			1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	*Z. intermedia* I	IP		0.015	0.001	0.063	0.002	0.000	0.000	0.024	0.000	0.000	0.005	0.011	0.002	0.001
2	*Z. intermedia* I	RS	**0.569**		0.010	0.100	0.013	0.000	0.000	0.108	0.002	0.001	0.024	0.020	0.014	0.008
3	*Z. intermedia* II	IP	**0.909**	**0.876**		0.050	0.003	0.000	0.000	0.022	0.000	0.000	0.004	0.005	0.001	0.000
4	*Z. intermedia* II	RS	0.959	0.904	**0.925**		0.474	0.000	0.000	0.327	0.020	0.017	0.067	0.043	0.039	0.028
5	*Z. intermedia* III	IP	**0.921**	**0.884**	**0.894**	0.143		0.000	0.000	0.035	0.000	0.000	0.004	0.005	0.004	0.000
6	*Z. intermedia* III	RS	**0.974**	**0.971**	**0.967**	**0.956**	**0.936**		0.316	0.000	0.000	0.000	0.000	0.000	0.000	0.000
7	*Z. gallii* II	RS	**0.952**	**0.947**	**0.941**	**0.916**	**0.889**	0.003		0.020	0.000	0.000	0.000	0.000	0.000	0.000
8	*Z. sango* II	IP	**0.954**	0.898	**0.919**	1.000	**0.842**	**0.864**	**0.743**		0.014	0.006	0.069	0.051	0.042	0.016
9	*Z. sango* II	RS	**0.974**	**0.962**	**0.960**	**0.997**	**0.961**	**0.978**	**0.961**	**0.997**		0.000	0.002	0.000	0.000	0.000
10	*Z. sango* II	CS	**0.980**	**0.972**	**0.968**	**1.000**	**0.969**	**0.980**	**0.963**	**1.000**	**0.888**		0.000	0.000	0.000	0.000
11	*Z. gallii* I	IP	**0.936**	**0.897**	**0.917**	0.939	**0.907**	**0.974**	**0.952**	0.934	**0.972**	**0.978**		0.003	0.005	0.003
12	*Z. gallii* I	RS	**0.974**	**0.955**	**0.954**	**1.000**	**0.948**	**0.979**	**0.960**	1.000	**0.998**	**1.000**	**0.738**		0.000	0.000
13	*Z. sango* I	IP	**0.968**	**0.949**	**0.949**	**0.996**	**0.945**	**0.976**	**0.955**	**0.995**	**0.995**	**0.998**	**0.953**	**0.996**		0.007
14	*Z. sango* I	RS	**0.967**	**0.950**	**0.951**	**0.988**	**0.948**	**0.976**	**0.956**	**0.987**	**0.990**	**0.993**	**0.952**	**0.989**	**0.471**	

Table 2.7. Updated host preference and distribution for all the coral-associated *Zanclea* species reported to date.

Species	Coral hosts	Localities	References
Z. gallii I	*Acropora*	Faafu Atoll (Maldives), Thuwal (Saudi Arabia), Bali and Tongian Island (Indonesia), Kenting and Penghu Island (Taiwan), Osprey Reef and Orpheus Island (Australia)	1, 7, 8, 11
Z. gallii II	*Acropora*	Dahab (Egypt), Eilat (Israel), Thuwal and Farasan Banks (Saudi Arabia)	1, 8, 12
Z. gillii	coral	Laing Island, Papua New Guinea	4
Z. intermedia I	*Cyphastrea*	Faafu Atoll (Maldives)	1, 10
	Gardineroseris	Faafu Atoll (Maldives), Thuwal (saudi Arabia)	
	Goniastrea	Faafu Atoll (Maldives)	
	Phymastrea	Faafu Atoll (Maldives)	
Z. intermedia II	*Montipora*	Faafu Atool (Maldives), Kenting (Taiwan), Dahab (Egypt), Thuwal (Saudi Arabia)	1, 7, 8, 9, 10
Z. intermedia III	*Porites*	Faafu Atoll (Maldives), Dahab (Egypt), Eilat (Israel), Thuwal and Farasan Banks (Saudi Arabia)	1, 8, 10
Z. margaritae	*Acropora*	Orpheus and Heron Island (Australia)	5
Z. sango I	*Pavona*	Okinawa (Japan), Faafu Atoll (Maldives), Dahab (Egypt), Ko Tao (Thailand)	1, 6, 8, 9, 10
	Psammocora	Okinawa (Japan)	
Z. sango II	*Coscinarea*	Thuwal (Saudi Arabia)	1, 8, 9, 10, 11, 12
	Cycloseris	Faafu Atoll (Maldives), Thuwal and Farasan Banks (Saudi Arabia)	
	Cyphastrea	Thuwal (Saudi Arabia), Eilat (Israel), Dahab (Egypt)	
	Danafungia	Faafu Atoll (Maldives), Farasan Banks (Saudi Arabia)	
	Dipsastrea	Faafu Atoll (Maldives), Eilat (Israel), Dahab (Egypt) Thuwal and Farasan Banks (Saudi Arabia)	
	Echinopora	Faafu Atoll (Maldives), Dahab (Egypt), Thuwal and Farasan Banks (Saudi Arabia)	
	Favites	Faafu Atoll (Maldives), Eilat (Israel), Dahab (Egypt) Thuwal (Saudi Arabia)	
	Fungia	Faafu Atoll (Maldives), Farasan Banks (Saudi Arabia)	
	Goniopora	Faafu Atoll (Maldives)	
	Halomitra	Faafu Atoll (Maldives)	
	Leptastrea	Faafu Atoll (Maldives), Eilat (Israel), Dahab (Egypt), Thuwal and Farasan Banks (Saudi Arabia)	
	Leptoseris	Faafu Atoll (Maldives), Thuwal and Farasan Banks (Saudi Arabia)	
	Orbicella	Sint Eustatius (Dutch Caribbean)	
	Pachyseris	Faafu Atoll (Maldives), Eilat (Israel), Thuwal and Farasan Banks (Saudi Arabia)	
	Pavona	Thuwal and Farasan Banks (Saudi Arabia)	

	Platygyra	Faafu Atoll (Maldives), Eilat (Israel), Dahab (Egypt) Thuwal (Saudi Arabia)	
	Pleuractis	Faafu Atoll (Maldives), Farasan Banks (Saudi Arabia)	
	Podabacia	Faafu Atoll (Maldives)	
	Psammocora	Faafu Atoll (Maldives), Dahab (Egypt), Thuwal and Farasan Banks (Saudi Arabia)	
	Stylocoeniella	Faafu Atoll (Maldives)	
	Symphyllia	Faafu Atoll (Maldives)	
	Turbinaria	Faafu Atoll (Maldives), Eilat (Israel)	
Zanclea sp.	coral	Inhaca Island, Mozambique	2, 3
Zanclea sp.	*Anacropora*	Lyudao and Kenting (Taiwan)	12
Zanclea sp.	*Astropora*	Kenting (Taiwan), Tongian Island (Indonesia)	12
Zanclea sp.	*Isopora*	Kenting (Taiwan)	12

1. This study, 2. Millard and Bouillon (1974), 3. Millard (1975), 4. Boero et al. (2000), 5. Pantos and Bythell (2010), 6. Hirose and Hirose (2011), 7. Fontana et al. (2012), 8. Montano et al. (2013), 9. Montano et al. (2014), 10. Montano et al. (2015a), 11. Montano et al. (2015b), 12. Pica et al. (2017).

2.8. FIGURES

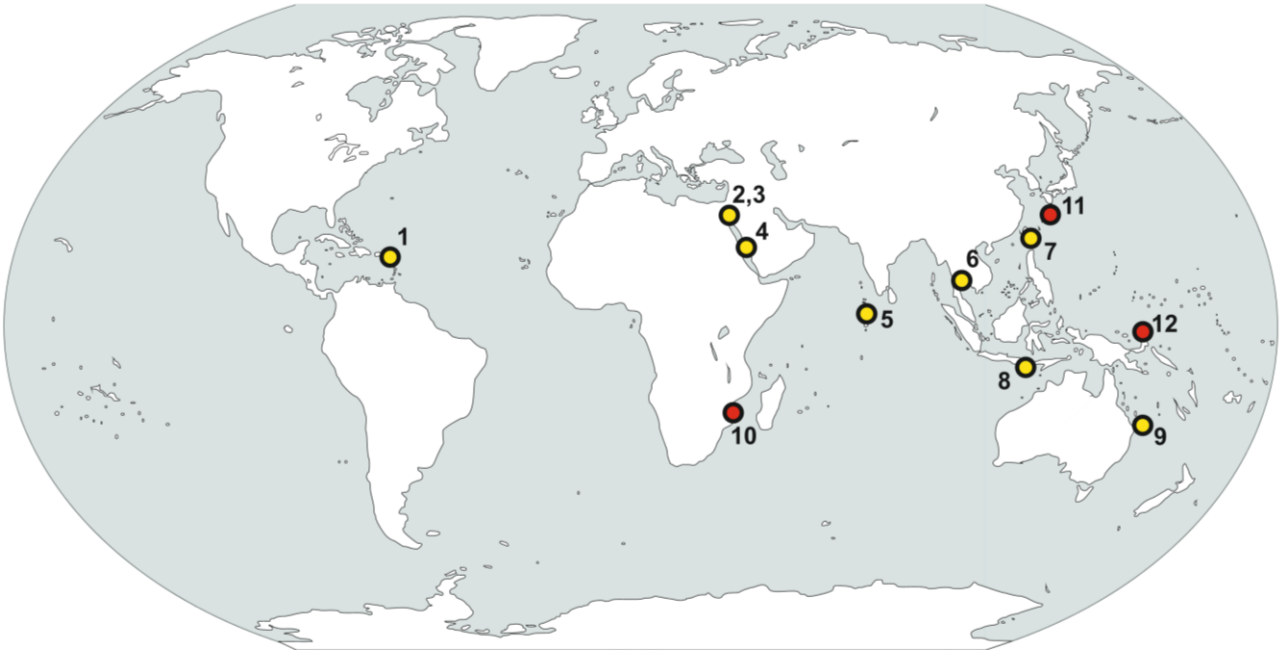

Figure 2.1. Known distribution of coral-associated *Zanclea*. Localities included in this work are indicated as yellow circles, and previous records as red circles. 1: Sint Eustatius (Dutch Caribbean); 2: Eilat (Israel); 3: Dahab (Egypt); 4: Thuwal and Farasan Banks (Saudi Arabia); 5: Faafu Atoll (Maldives); 6: Ko Tao (Thailand); 7: Kenting and Penghu Island (Taiwan); 8: Bali (Indonesia); 9: Great Barrier Reef (Australia); 10: Inhaca Island (Mozambique); 11: Okinawa (Japan); 12: Laing Island (Papua New Guinea).

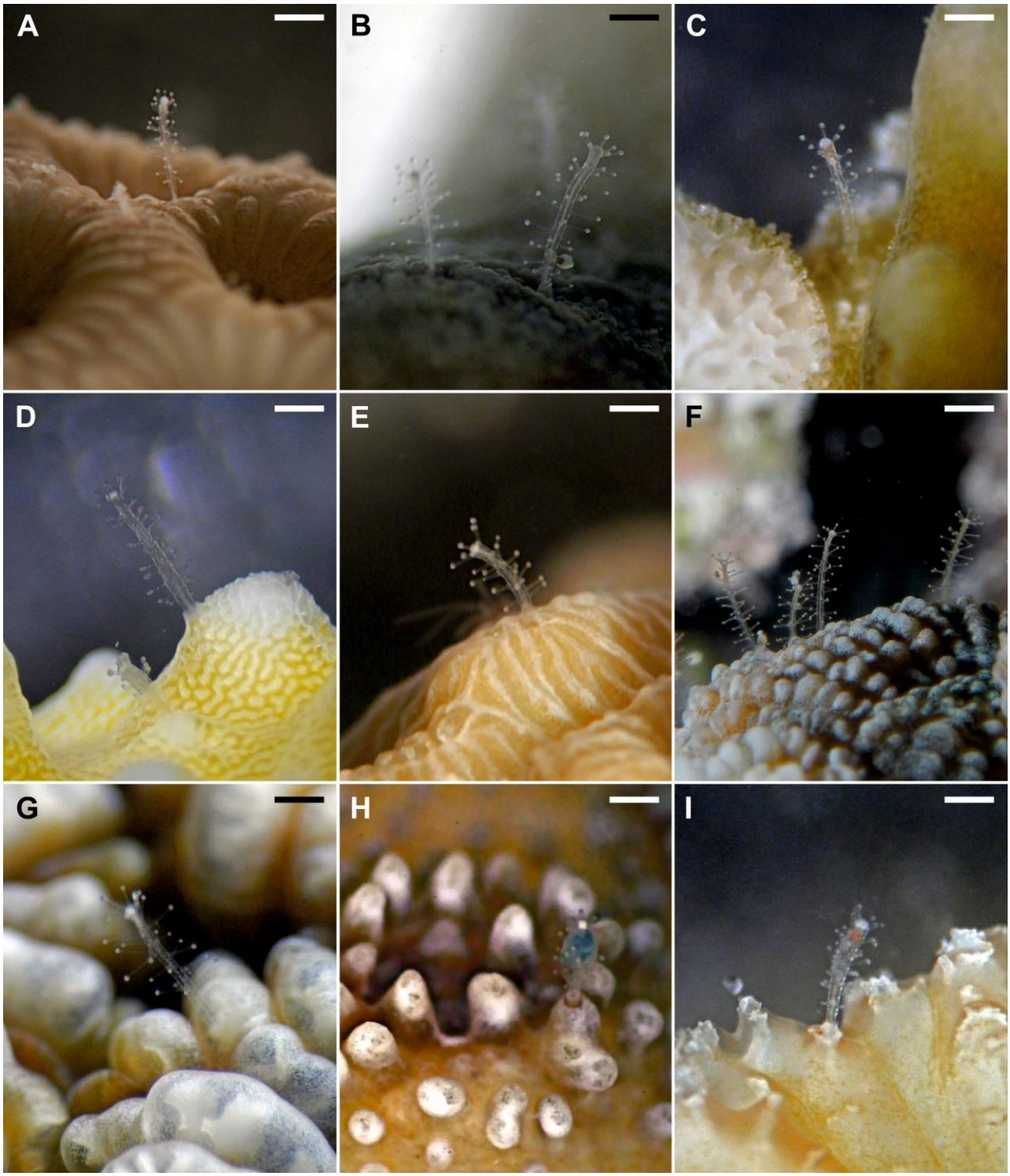

Figure 2.2. In situ photographs and microphotographs of living *Zanclea* polyps associated with A) *Goniastrea*, B) *Porites*, C) *Montipora*, D) *Acropora*, E) *Pavona*, F) *Favites*, G) *Dipsastrea*, H) *Echinopora*, I) *Platygyra*. Scale bars: ~ 500μm.

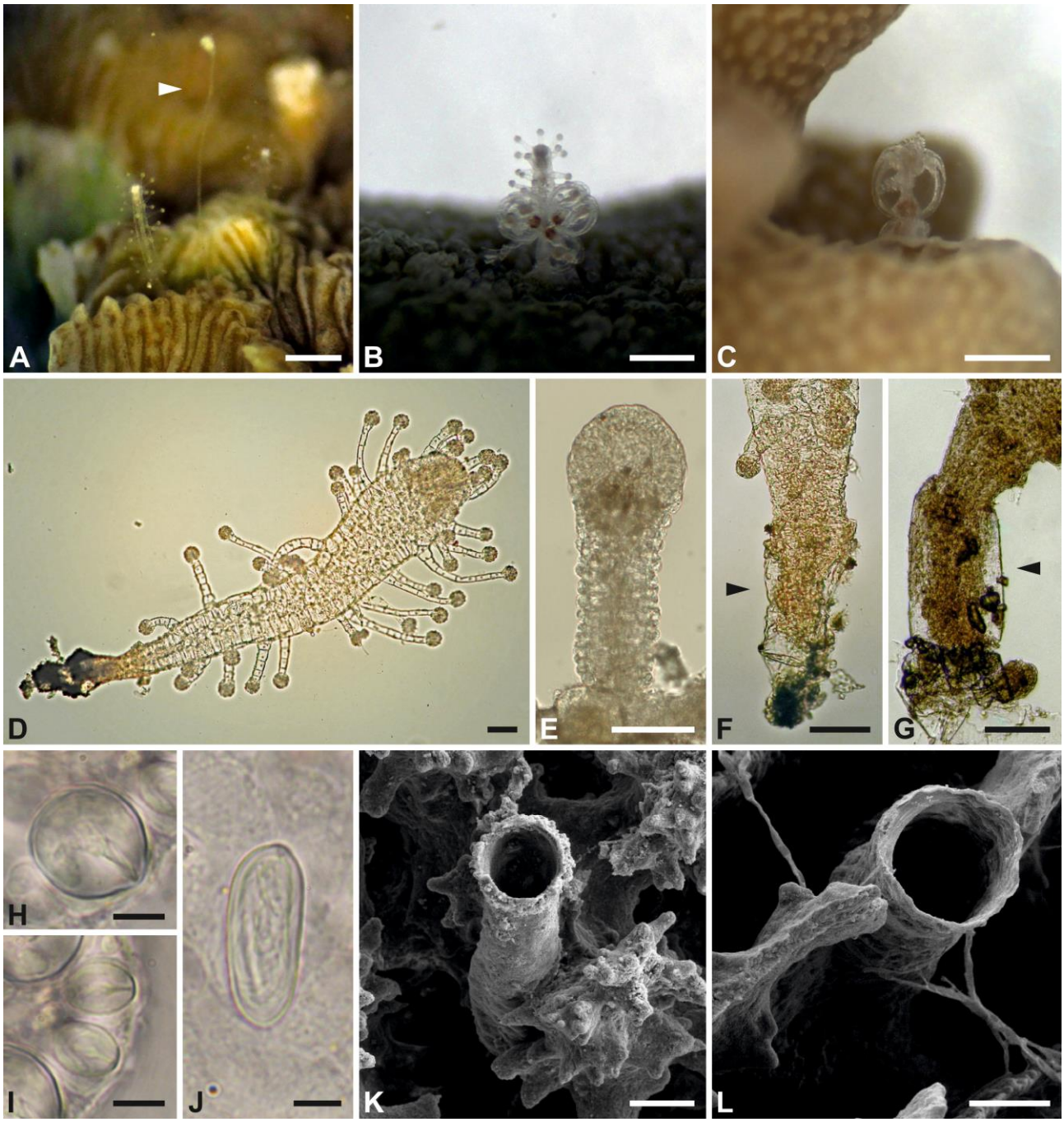

Figure 2.3. Micrographs of the polyp stage of *Zanclea* associated with various corals. A) Polymorphic colony on *Pavona*. B) Fertile polyp on *Porites* with at least three medusa buds. C) medusa bud arising directly from the hydrorhiza on *Acropora*. D) Detached gastrozooids collected from *Leptastrea*. E) Dactylozooid collected from *Pavona*. F, G) Detail of polyps collected from *Leptoseris* and *Leptastrea*, respectively, with the basal part covered by a transparent perisarc (arrowheads). H, I, J), Large and small stenetoles, and eurytele, respectively, belonging to a polyp collected from *Goniastrea*. K, L) SEM images of the coral skeleton overgrowing the basal part and the hydrorhiza of polyps growing on *Porites* and *Pavona*, respectively (tissues removed). Scale bars: A-C) 0.5 mm, D-G) 100 μm, K, L) 50 5μm, H-J) 5μm.

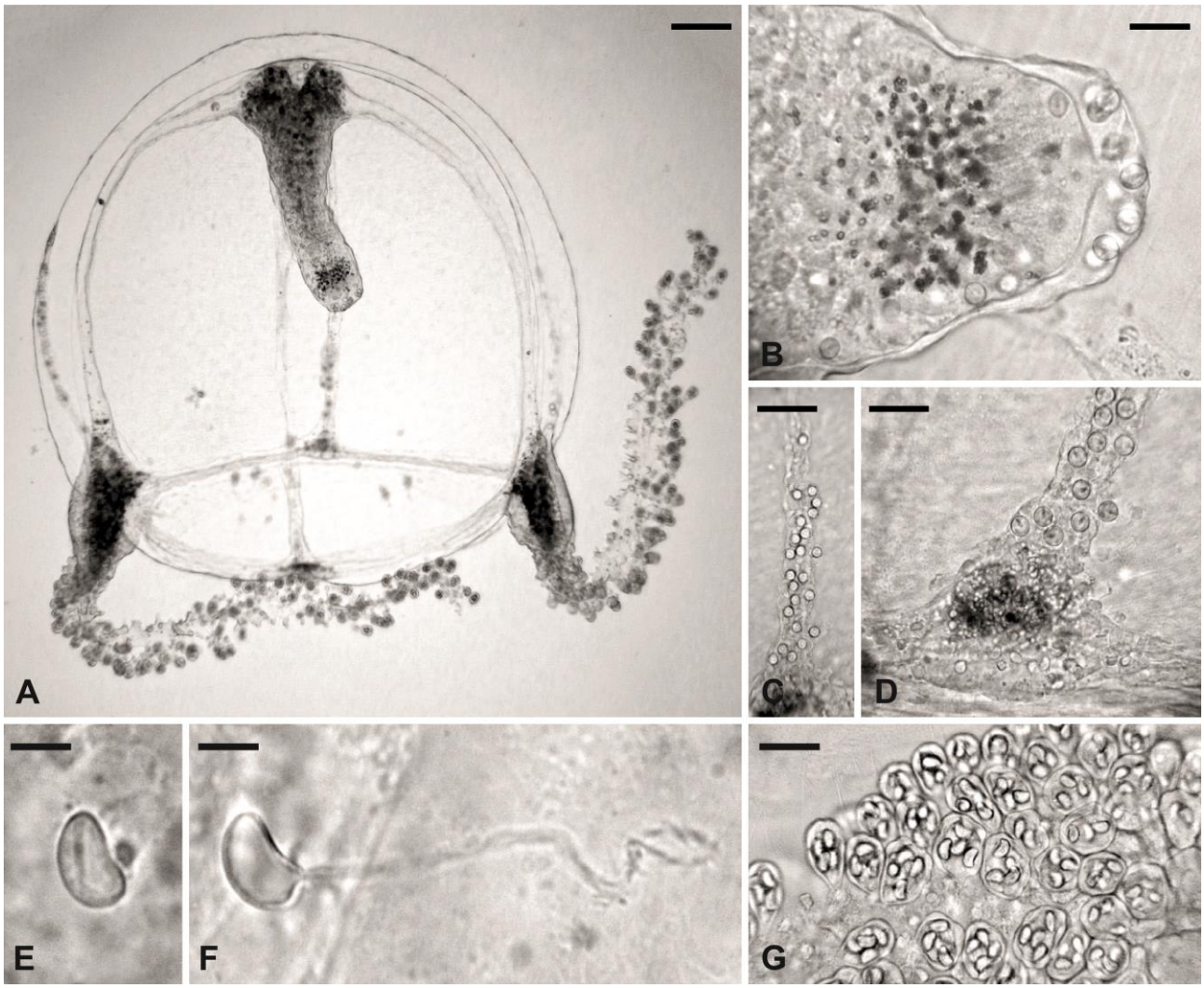

Figure 2.4. Micrographs of the medusa stage of the *Acropora*-associated *Zanclea gallii*. A) One day old medusa. B) Mouth surrounded by small stenoteles. C) Exumbrellar nematocyst pouch with large stenoteles. ending in a D) small bulb without tentacle. E) Undischarged and F) discharged bean-shaped macrobasic euryteles found in the tentacular bulb and in G) the cnidophores. Scale bars: A) 100 μm, B) 25 μm, C) 50 μm, D) 35 μm, E, F) 4 μm, G) 45 μm.

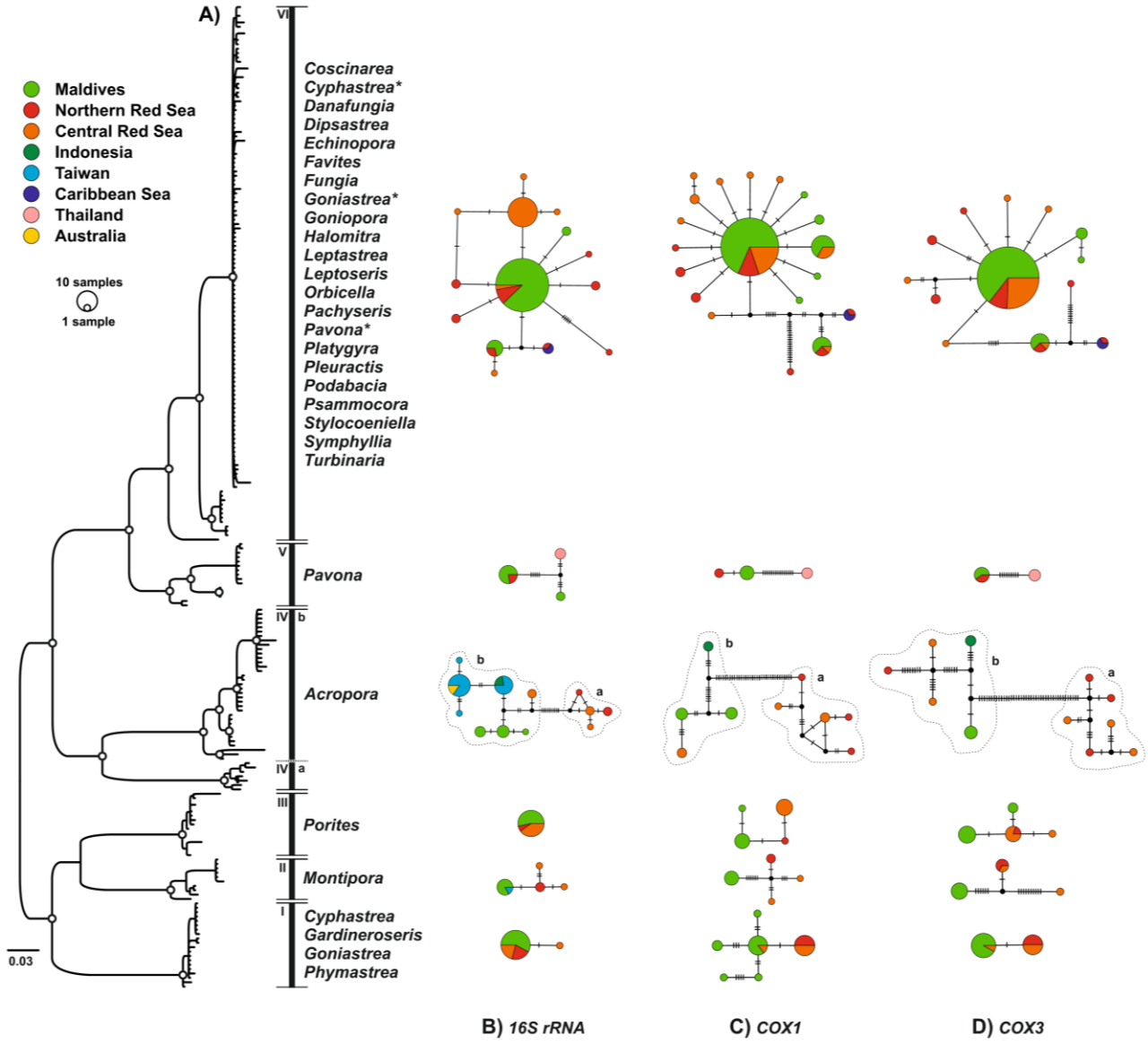

Figure 2.5. Phylogenetic tree and haplotype network analyses based on mitochondrial genes. A) Bayesian phylogenetic tree inferred from the mitochondrial concatenated dataset. B, C, D) Most parsimonious median-joining single-locus mitochondrial haplotype networks for the *16S*, *COX1*, and *COX3*, respectively. In haplotype networks, the size of circles is proportional to the frequencies of specimens sharing the same haplotype, each colour represents a different sampling locality, black circles represent hypothetical intermediate haplotypes, and dashes indicate the number of substitutions. Asterisks in the phylogenetic tree (Clade VI) indicate that corals host also other *Zanclea* clades.

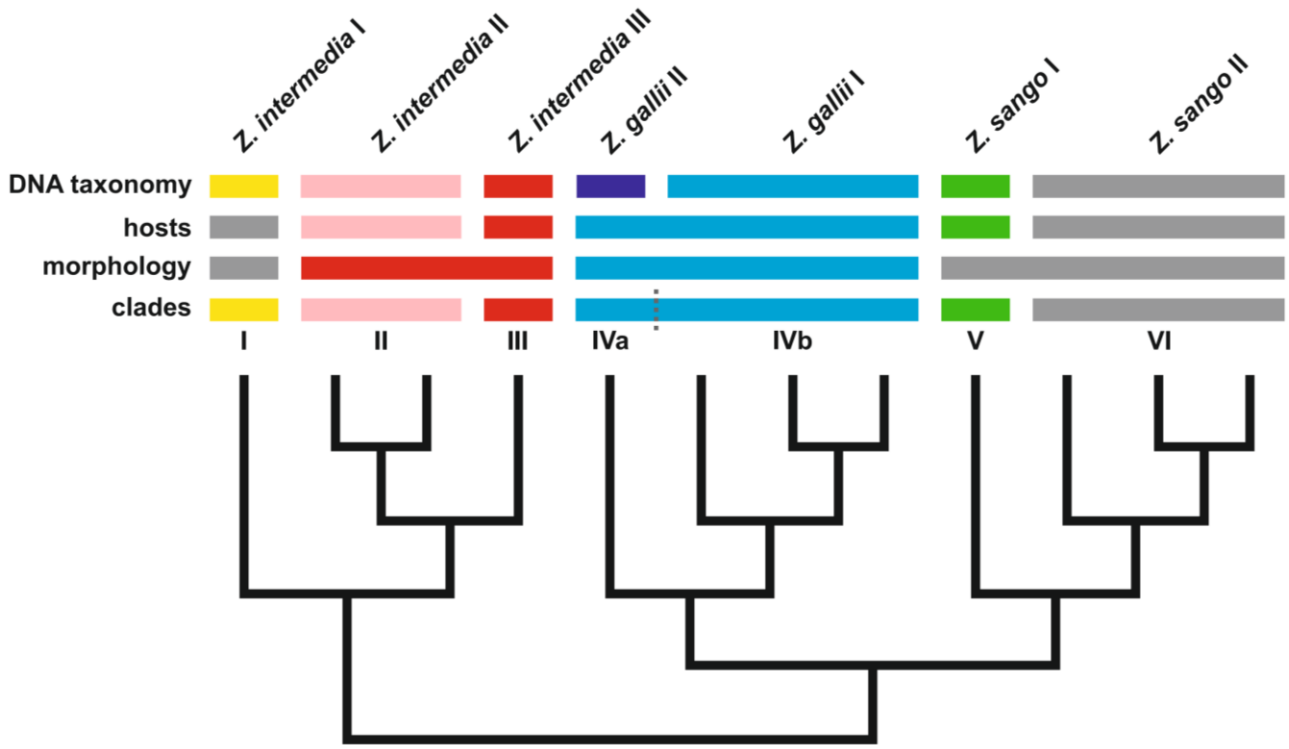

Figure 2.6. Simplified cladogram of coral-associated *Zanclea* showing the species delimitation based on species delimitation techniques (DNA taxonomy), host preference, morphology, and phylogenetic clades.

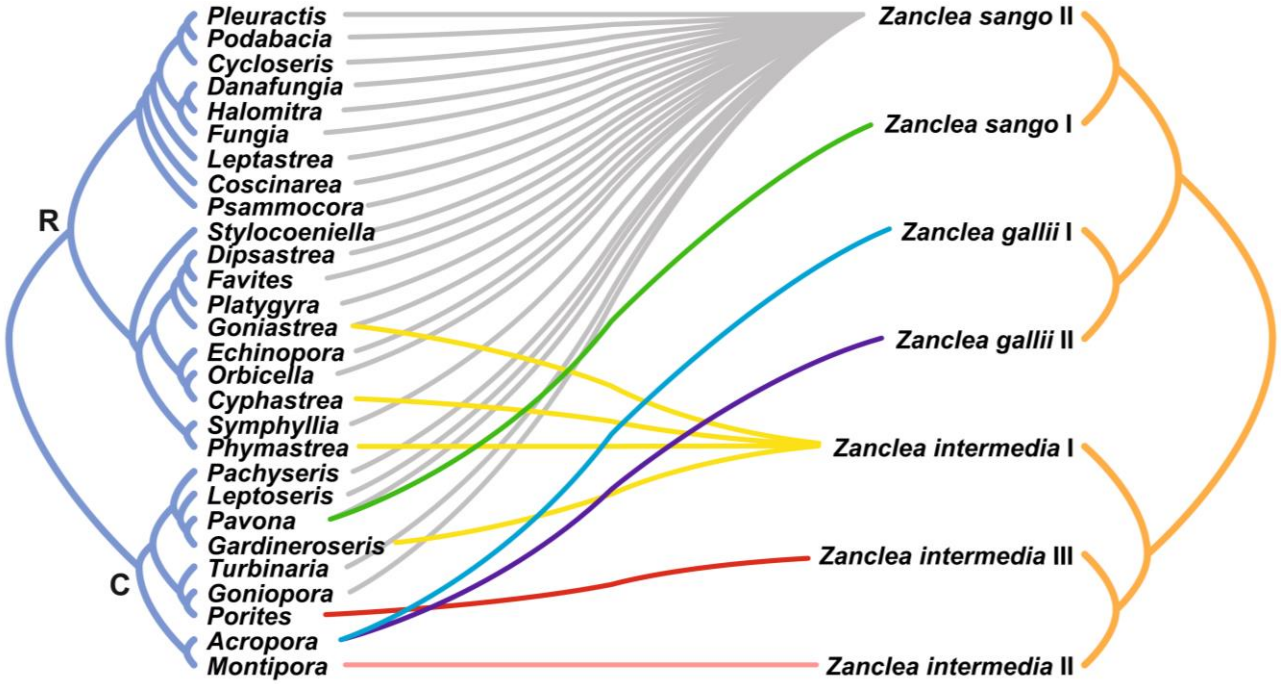

Figure 2.7. Tanglegram showing the associations between scleractinian and *Zanclea* terminals. Colours of the links refer to those used in the species delimitation in Figure 2.6. C: clade 'Complexa', R: clade 'Robusta'.

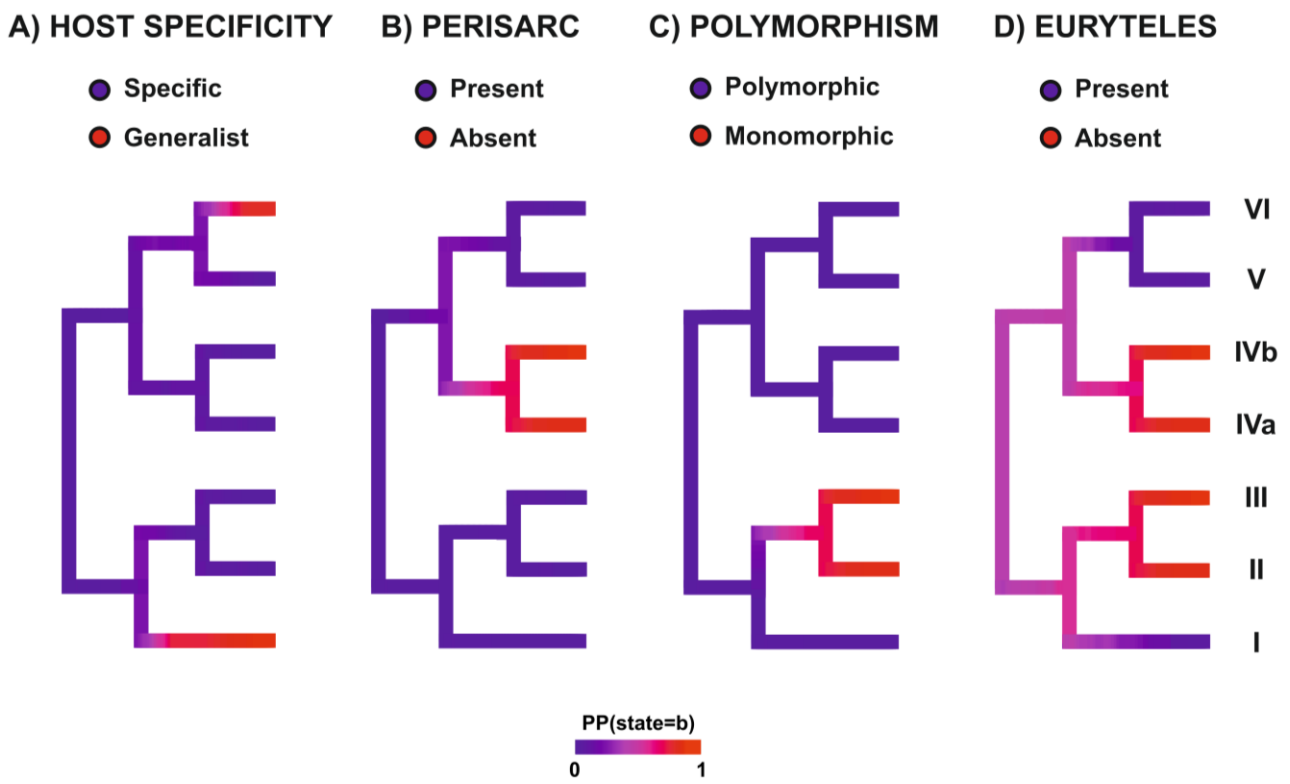

Figure 2.8. Density plot of 1000 stochastic character maps of the characters A) host specificity (0: specific, 1: generalist), B) Perisarc (0: present, 1: absent), C) Polymorphism (0: polymorphic colonies, 1: monomorphic colonies), D) Euryteles (0: present, 1: absent). The colour of edges in the trees gives the posterior probability (computed as the relative frequency across stochastic maps) of each character state. Red indicates high posterior probability of generalism, absence of perisarc, polymorphism and euryteles.

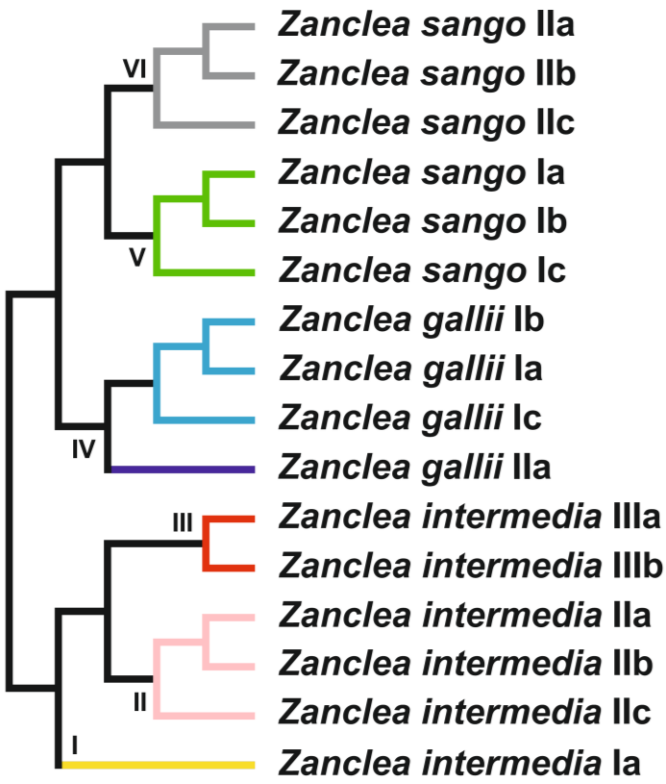

Figure 2.S1. Summary of the names given to each recovered clade following the nomenclature proposed by Morard et al. (2016). Roman numerals on the tree correspond to the phylogenetic clades as shown in Figure 2.5.

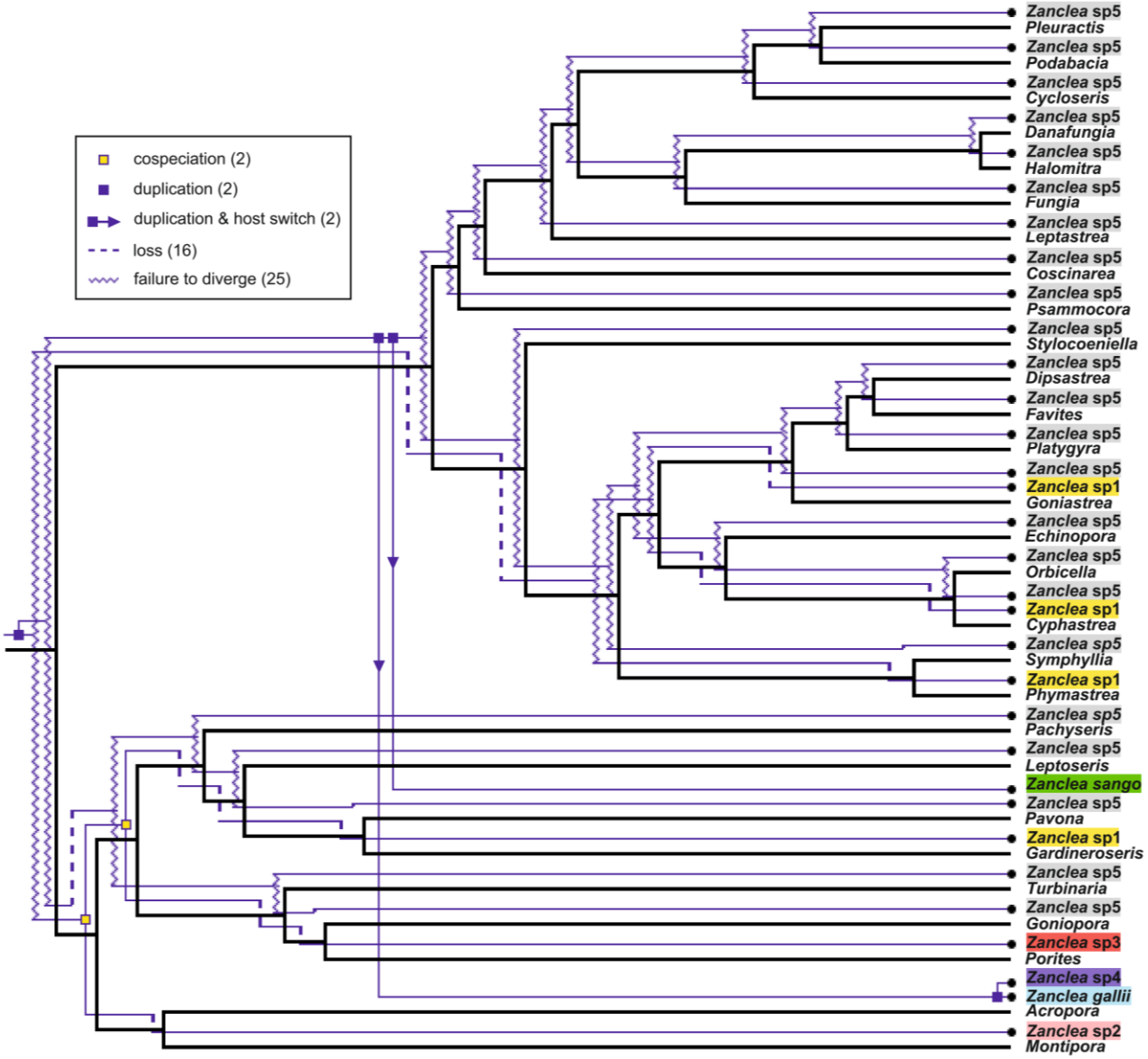

Figure 2.S2. Trees resulting from the analysis in Jane 4.0 showing the different coevolutionary events between scleractinian corals (black lines) and coral-associated Zanclea (blue lines). Different *Zanclea* species are highlighted with the same colours as in Figure 2.6.

CHAPTER 3

The *Pteroclava krempfi* species complex and its allies: a morpho-molecular assessment of the family Cladocorynidae (Hydrozoa, Capitata)

This work is partially published in:

1. Seveso D, Montano S, Pica D, Maggioni D, Galli P, Allevi V, Bastari A, Puce S (2016). *Pteroclava krempfi*-octocoral symbiosis: new information from the Indian Ocean and the Red Sea. *Marine Biodiversity* 46, 483-487. doi: 10.1007/s12526-015-0368-y.
2. Maggioni D, Montano S, Seveso D, Galli P (2016). Molecular evidence for cryptic species in *Pteroclava krempfi* (Hydrozoa, Cladocorynidae) living in association with alcyonaceans. *Systematics and Biodiversity* 14, 484-493. doi: 10.1080/14772000.2016.1170735.
3. Montano S, Maggioni D, Galli P, Hoeksema BW. (2017). A cryptic species in the *Pteroclava krempfi* species complex (Hydrozoa, Cladocorynidae) revealed in the Caribbean. *Marine Biodiversity* 47, 83-89. doi: 10.1007/s12526-016-0555-5.

and in preparation for the submission as:

4. Maggioni D, Galli P, Seveso D, Berumen ML, Arrigoni R, Hoeksema BW, Montano S. The *Pteroclava krempfi* species complex and its allies: a morpho-molecular assessment of the family Cladocorynidae (Hydrozoa, Capitata).

3.1. ABSTRACT

The hydrozoan *Pteroclava krempfi* is a widespread species known to be mainly associated with alcyonacean octocorals in the Indo-Pacific and Caribbean. In this study, the host range as well as the distribution of this species is widened and the morphological and genetic diversity are investigated. *Pteroclava krempfi* harbours a high genetic diversity despite a conserved morphology and, according to molecular phylogenetics and DNA taxonomy analyses, four cryptic species can be identified. Each species has a different host preference and distribution range and, in some cases, a further genetic structuring is attributable to geographic diversification. With this work, the first molecular phylogeny of the family Cladocorynidae, to which *Pteroclava* belongs, is also presented. The other known cladocorynid genus is *Cladocoryne*, for which two out of five species were analysed and, in one species (*Cladocoryne floccosa*), the high intra-specific genetic diversity may be related to the presence of cryptic or pseudocryptic species. Additionally, morphological assessments and molecular phylogenetics allowed the establishment of a new genus here called *Pseudozanclea*. This genus is here erected to accommodate the species *Pseudozanclea timida*, previously ascribed to the genus *Zanclea* and family Zancleidae. Overall, this study highlights that alcyonacean octocorals host a higher diversity of hydrozoan associates than previously known, similarly to scleractinian corals, and that this association can be found in several localities. Moreover, the family Cladocorynidae is demonstrated to include a previously unsuspected taxon and to possibly contain a further cryptic diversity also in the *Cladocoryne* species.

3.2. INTRODUCTION

Interspecific associations are very common in coral reef ecosystems and often involve small animals that live as endobionts in the skeletons of corals, as epibionts on their surface or in between their tentacles (Patton 1972, Goh et al. 1999, Stella et al. 2011, Hoeksema et al. 2012). Hence, these corals construct a multitude of habitats for a diverse cryptobenthic fauna estimated to be over 172,000 species, belonging to a multitude of phyla (Reaka-Kudla et al. 1996, Ruppert et al. 2004). However, invertebrates have received little attention, even though they account for the vast majority of animal species on coral reefs (Stella et al. 2011, Fisher et al. 2015). Moreover, many nominal species of invertebrates contain a cryptic diversity that can be unveiled only by using molecular techniques (Knowlton 2000), making the potential number of species living in tropical ecosystems such as coral reefs higher than previously estimated (Bickford et al. 2007, Fisher et al. 2015). Research on cryptic species has grown dramatically over the last three decades, and the increasing availability of DNA sequences has allowed taxonomists to unravel the hidden genetic diversity of several taxa (Bickford et al. 2007, Appeltans et al. 2012). This is also the case for hydrozoans, among which cryptic species seem to be common, especially for those showing few distinctive morphological features (e.g. Folino-Rorem et al. (2009), Miglietta et al. (2009), Moura et al. (2012a)). Hydrozoans can live in symbiosis with several other organisms, including other cnidarians, such as scleractinian corals and octocorals (Puce et al. 2008a). Regarding octocorals, most of their sessile associates usually grow on dead portions of the colony (Love et al. 2007), whereas some other, including hydrozoans, are intimately associated with the living parts (Puce et al. 2008a). To date, 11 species of hydrozoans are known to live in specific associations with at least 16 species of octocorals (Gili et al. 2006, Puce et al. 2008b, Bo et al. 2011). All these hydrozoans belong to the order Anthoathecata, and are representative of five families: Asyncorynidae Kramp 1949, Cladocorynidae Allman 1872, Corynidae Johnston 1836, Tubulariidae Goldfuss 1818, and Zancleidae Russle 1953. Among these hydrozoans, the circumtropical species *Pteroclava krempfi* (Billard, 1919) is associated with three alcyonacean genera belonging to two families, namely *Cladiella* Gray 1869 (family Alcyoniidae Lamouroux 1812), *Astrogorgia* Verril 1868, and *Plexaurella* Kölliker 1865 (family Plexauridae Gray 1859), plus other unidentified alcyonaceans and gorgonians (Billard 1919, Weill 1931, Hirohito 1988, Boero et al. 1995, Puce et al. 2008b, Varela 2010). *Pteroclava krempfi* was originally recorded in Vietnam (Billard 1919), and was subsequently reported from Japan (Hirohito 1988), Papua New Guinea, La Réunion (Boero et al. 1995), Indonesia (Puce et al. 2008b) and Cuba (Varela 2010). *Pteroclava krempfi* is congeneric with *Pteroclava crassa* Pictet 1893, which was described from Indonesia (Pictet 1893) and strongly resembles *P. krempfi*: according to Boero et al. (1995) the main difference is the host, being *P. crassa* associated with the hydrozoan *Macrorhynchia philippina* Kirchenpauer 1872.

The main morphological features of both *Pteroclava* species are the moniliform tentacles and the eurytele patches in the polyp body walls (Bouillon et al. 2006). The medusa stage is known only for *P. krempfi* and the most distinctive characteristic is the presence of two exumbrellar pouches containing eurytele capsules (Boero et al. 1995). The genus *Pteroclava* Weill 1931 was firstly placed in the Cladocorynidae by Petersen (1990),

whose definition of the family contained many morphological imprecisions. Afterwards, Boero et al. (1995) clarified the definition of this family and provisionally accepted the inclusion of *Pteroclava* in the Cladocorynidae family. To date, no molecular study included *Pteroclava* specimens and therefore the correct placement into the Capitata suborder and the genetic diversity of the genus still remain to be investigated.

The only other genus in the family Cladocorynidae is *Cladocoryne* Rotch 1871, which counts five species: the type species *Cladocoryne floccosa* Rotch 1871, with a worldwide distribution in tropical, subtropical, and temperate waters (see Nagale and Apte (2014) and herein references); *Cladocoryne haddoni* Kirkpatrick 1890, reported from several localities in the Indian and Pacific Ocean (Schuchert 2003); *Cladocoryne littoralis* Mammen 1963 and *Cladocoryne travancorensis* Mammen 1963, described and exclusively found in Indian waters (Mammen 1963); and *Cladocoryne minuta* Watson 2005, from Australia (Watson 2005). The main character for discriminating among *Cladocoryne* species is the type and arrangement of aboral tentacles (Schuchert 2003). Specifically, *C. floccosa*, *C. haddoni*, and *C. littoralis* are characterised by three, two, and one whorls of ramified capitate tentacles, respectively, *C. travancorensis* has three whorls of cateniform tentacles, and *C. minuta* has two whorls of tentacles with a terminal capitulum and three or four opposite pairs of short capitate side branches (Kirkpatrick 1890, Mammen 1963, Watson 2005). The taxonomy of the genus is however still not fully elucidated. For instance, Bouillon et al. (1987) synonymised the genera *Lobocoryne* Mammen 1963 (*Lobocoryne travancorensis*) and *Cladocorynopsis* Mammen 1963 (*Cladocorynopsis littoralis*) with *Cladocoryne*, but these two taxa were never found after their description by Mammen (1963) and also their reproductive structures are unknown. Moreover, Schuchert (2003) hypothesised the presence of still undescribed species of *Cladocoryne*, based on the observation of the number and arrangement of tentacles.

The family Cladocorynidae is characterised by the presence of large macrobasic apotrichous eurytele capsules, grouped in rounded patches and in some cases scattered at the base of tentacles (Bouillon et al. 2006). Similar nematocysts are found in the octocorals-associated *Zanclea timida* Puce, Di Camillo & Bavestrello 2008 (Puce et al. 2008b), in which a band of nematocysts (in this case apotrichous macrobasic mastigophores) are found at the base of hydranths. This species is only reported from Indonesia and no reproductive structures have been observed, leaving nevertheless some doubts regarding the assignment to the genus *Zanclea* Gegenbaur 1856.

The present study aims at i) clarifying the phylogenetic relationships within the Cladocorynidae, ii) assessing the morphological and molecular diversity and relationships of *P. krempfi*, and iii) re-evaluate the morphological features and the phylogenetic position of *Zanclea timida*.

3.3. MATERIAL AND METHODS

3.3.1. Sample Collection and Morphological Analyses

Samples included in the analyses were collected in field surveys conducted from March 2014 to May 2017 at Eilat (Israel), Dahab (Egypt), Thuwal, and Farasan Banks (Saudi Arabia) in the Red Sea; Faafu Atoll (Maldives) in the Indian Ocean; Bali and Komodo (Indonesia) in the Pacific Ocean; Sint Eustatius (Netherlands) and Bocas del Toro (Panama) in the Caribbean Sea. Moreover, additional DNA extracts of

Cladocoryne floccosa sampled in the Caribbean and Mediterranean Sea were obtained from the Natural History Museum of Geneva.

Cladocorynid colonies were collected with hammer and chisel or a knife. When associated with biotic substrates (i.e. octocorals), a fragment of the host bearing hydrozoans was collected. Hydrozoan colonies were anesthetised with menthol crystals, and single hydrozoan polyps were carefully collected one by one using syringe needles, precision forceps, and micropipettes directly from a bowl filled with seawater placed under a stereomicroscope. Part of the detached polyps were directly observed at the microscope, whereas others were immediately preserved in 95 % ethanol and 10 % formalin for further molecular and morphological analyses, respectively. Portions of the colonies were also placed in small oxygenated water bowls and were cultured for few days. When medusae liberation occurred, the medusae were maintained in small bowls (seven days for *Z. timida* and three days for *P. krempfi*) at ambient temperature and fed *Artemia* nauplii. The water was replaced two hours after feeding every day. The reared medusae were observed on a daily basis, and some medusae were fixed in 10% formalin.

Morphological observations, pictures and measurements of the polyps, medusae and nematocysts were mainly performed using living specimens. Underwater photographs were taken using a Canon G11 camera in a Canon WP-DC 34 underwater housing. Microphotographs of hydroids, medusae, and nematocysts were taken using a Leica EZ4 D stereomicroscope and a Zeiss Axioskop 40 microscope both equipped with a Nikon AW 100 camera and ocular micrometrics.

Hydrozoans were identified to the species level according to Bouillon et al. (1987), Boero et al. (1995), and Puce et al. (2008b), and octocorals were identified to the genus level using Fabricius and Alderslade (2001) and Williams and Chen (2012).

3.3.2. Molecular Analyses

The total genomic DNA of ethanol-fixed samples was extracted following a protocol modified from Zietara et al. (2000). Seven different molecular markers were amplified: i) a ~600 bp portion of the mitochondrial *16S* ribosomal DNA gene (*16S*), ii) a ~700 bp portion of the mitochondrial cytochrome oxidase subunit I gene (*COX1*), iii) a ~700 bp portion of the mitochondrial cytochrome oxidase subunit III gene (*COX3*), iv) a ~1700 bp portion of the nuclear *18S* ribosomal DNA gene (*18S*), v) a ~1700 bp portion of the nuclear *28S* ribosomal DNA gene (*28S*), vi) a ~700 bp portion of the nuclear internal transcribed spacer ribosomal region (*ITS*), and vii) a ~400 portion of the histone H3 gene (*H3*). *16S, COX3, 28S,* and *ITS* regions were amplified using hydrozoan-specific primers and the protocols proposed by Cunningham and Buss (1993), Peña-Cantero and Sentandreu (2017), Maggioni et al. (2016), and Fontana et al. (2012), respectively. *COX1*, *18S*, and *H3* genes were amplified using universal primers and the protocols proposed by Folmer et al. (1994), Medlin et al. (1988), and Colgan et al. (1998), respectively. All PCR products were purified with Illustra ExoStar (GE Healthcare) at 37° for 60 min, followed by 85° for 15 min and then directly sequenced in forward and reverse directions using an ABI 3730xl DNA Analyzer (Applied Biosystems). The obtained chromatograms were visually checked and assembled using Sequencher 4.1.4 (Gene Codes). *COX1, COX3, H3* sequences were

translated in Geneious 6.1.6 (Drummond et al. 2010), in order to check for the presence of stop codons. Sequences of each marker were aligned with MAFFT v. 7.110 (Katoh and Standley 2013) using the E-INS-i option and *16S*, *18S*, *28S*, and *ITS* alignment were run through Gblocks (Castresana 2000, Talavera and Castresana 2007) using the default 'less stringent' settings in order to remove ambiguously aligned regions. Phylogenetic inference analyses were performed for all single locus datasets and for the concatenated dataset using Bayesian inference (BI) and maximum likelihood (ML). Appropriate partition schemes and models were determined using PartitionFinder 1.1.1 (Lanfear et al. 2012) by means of the Akaike Information Criterion (AIC). BI analyses were performed using MrBayes 3.2 (Ronquist et al. 2012). Four parallel Markov Chain Monte Carlo runs (MCMC) were run for 10^7 generations for the each dataset. Trees were sampled every 100^{th} generation and burn-in was set to 25%, based on checking the parameter estimates and convergence using Tracer 1.6 (Rambaut et al. 2014). Maximum likelihood trees were built with Garli 2.01 (Zwickl 2006) and read into the SumTrees 4.0.0 program in the DendroPy 4.0.0 package (Sukumaran and Holder 2010) to calculate non-parametric bootstrap support (BS) values from 1000 replicates, each based on five heuristic search replicates, and to map them on the best ML tree. Single-locus ultrametric trees were built for *Pteroclava* species delimitation analyses using Beast 1.8.2 (Drummond et al. 2012), using a relaxed log-normal clock with a coalescent tree prior: MCMC were run for $5x10^7$ generations, sampling every 1000 generations, chain convergence was assessed using Tracer 1.6 (Rambaut et al. 2014), and the consensus trees (with 25% burn-in) were built with TreeAnnotator 1.7 (Rambaut and Drummond 2013).

Genetic distances (uncorrected p-distance, 1000 bootstrap) within and among the main molecular lineages were computed for each separated molecular locus using MEGA 6 (Tamura et al. 2013).

To determine molecular species in the *Pteroclava krempfi* complex, three independent species delimitation approaches were used for each single locus dataset: the Automatic Barcoding Gap Discovery (ABGD), the Poisson-Tree-Processes (PTP), and the Generalized Mixed Yule Coalescent (GMYC). ABGD analyses (Puillandre et al. 2012) were run on the web server http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html. The alignments were imported in MEGA 6.0 (Tamura et al. 2013) to compute matrices of pairwise genetic distances using the Kimura 2-parameter (K2P), the p-distance, and the Jukes-Cantor (JC69), and parameters were set as follows: Pmin = 0.001, Pmax = 0.05, Steps = 50, X = 1.5, and Nb bins = 20. PTP and bPTP analyses (Zhang et al. 2013) were performed on the web server http://species.h-its.org/ptp/, using the 50% majority-rule consensus topology resulting from Bayesian analyses. PTP analyses were run for $5x10^5$ MCMC generations, with thinning value = 100 and burn-in = 0.25. Clusters with a probability $\geq$ 0.9 were considered as corresponding to species. Finally, ultrametric trees were used to perfom single-threshold (stGMYC) (Pons et al. 2006), multiple-threshold (mtGMYC) (Monaghan et al. 2009), and Bayesian (bGMYC) (Reid and Carstens 2012) GMYC analyses in R 3.1.3 (R Core Team, 2013) using the packages 'Splits' (Ezard et al. 2009), 'Ape' (Paradis et al. 2004) and 'bGMYC' (Reid and Carstens 2012). For bGMYC analysis, clusters with a probability $\geq$ 0.9 were considered as successfully delimited species.

CAOS software was used to identify diagnostic nucleotides for the newly recovered species (Sarkar et al. 2002, Sarkar et al. 2008, Bergmann et al. 2009), following the instructions of Jörger and Schrödl (2013). Only single

pure character attributes were considered as diagnostic characters, which are single nucleotides present in all members of a clade identified as a species, but absent in members of other clades (Jörger and Schrödl 2014). The nomenclatural system recently proposed by Morard et al. (2016) was then used to name the recovered cryptic clades.

For the species with a distribution not limited to a single region, genetic differentiation among populations was tested using analysis of molecular variance (AMOVA) of *16S rRNA* sequences (most complete dataset) in Arlequin 3.5.1.2 (Excoffier and Lischer 2010).

3.4. RESULTS

The field surveys allowed the collection of 53 colonies of *Pteroclava krempfi* from all the investigated localities, seven colonies of *Cladocoryne haddoni* from Maldives, and six colonies of *Zanclea timida* from Maldives. *Pteroclava krempfi* was found in association with eight octocoral genera, namely *Sarcophyton* Lesson 1834, *Lobophytum* Marenzeller 1886, *Sinularia* May 1898, *Rhytisma* Alderslade 2000 (family Alcyoniidae), *Paraplexaura* Kükenthal 1909, *Astrogorgia* (family Plexauridae), *Antillogorgia* Bayer 1951 (family Gorgoniidae Lamouroux, 1812), and *Melithaea* Milne Edwards 1857 (family Melithaeidae Gray 1870). *Cladocoryne haddoni* was found on different biotic and abiotic substrates, including rock, coralline algae, ascidians, and sponges. Finally, *Z. timida* was found growing on the octocoral *Bebryce* sp. (family Plexauridae) and in association with an undetermined overgrowing sponge. Most of the colonies were collected at shallow water (0-20 m deep), with the exception of *P. krempfi* associated with *Paraplexaura* from Maldives (40 m deep) and *Z. timida* (45 m deep).

Morphological and molecular analyses revealed that all these species, including *Z. timida*, belong to the family Cladocorynidae, and that *Pteroclava krempfi* is a complex of cryptic species. The morphology of all these species is described in the following 'Morphological Analyses' paragraph, whereas the molecular diversity and phylogenetic relationships results are described in the 'Molecular Analyses' paragraph.

3.4.1. Morphological Analyses

Family Cladocorynidae Allman 1872

Genus *Cladocoryne* Rotch 1871

Cladocoryne haddoni Kirkpatrick 1890

Figure 3.1

<u>*Polyp*</u>: Colonies growing on a variety of substrates, including rock, coralline algae, ascidians, and sponges. Stems up to 1.5 cm long, originating from perisarc-covered hydrorhiza. Perisarc stopping at the base of polyp. Polyps up to 2 mm long with an oral whorl of 4-6 adnate capitate tentacles and two whorls of 6-8 ramified capitate tentacles (Figure 3.1A-D). Three to five patches of euryteles in the area between oral and aboral

tentacles (Figure 3.1C, F). Smaller euryteles at the base of some proximal aboral tentacles (Figure 3.1G). Polyps and gonophores transparent with reddish gastroderm and manubrium.

Gonophore: Gonophores cryptomedusoid (only males observed) (Figure 3.1B, E), about 1 mm long, up to two per hydranth and borne on pedicels in the area between oral and aboral tentacles under eurytele patches. Four rudimentary perradial canals ending in highly reduced tentacular bulbs with no tentacles, and a rudimentary circular canal. Male manubrium large, bearing a white mass of sperm.

Cnidome: i) Small stenoteles in tentacles (8-9 x 5-714-17 x 11-12 μm). ii) Large stenoteles in tentacles and, rarely, on cryptomedusoids (14-17 x 11-12 μm). iii) Large apotrichous macrobasic euryteles in patches below oral tentacles, scattered below aboral tentacles, on cryptomedusoids (48-55 x 20-23 μm; discharged shaft: 720-840 μm). iv) Small apotrichous macrobasic euryteles at the base of proximal aboral tentacles (25-28 x 18-20 μm; discharged shaft: 202-230 μm).

Remarks: All the investigated colonies clearly correspond to *Cladocoryne haddoni*. Bouillon et al. (1987) found diagnostic characters to easily separate this species from the similar *Cladocoryne floccosa*. Specifically, the presence of two whorls of about eight aboral tentacles and the small euryteles at the base of proximal tentacles allow to identify also young and immature polyps.

Genus *Pseudozanclea* gen. nov.

Pseudozanclea timida (Puce, Di Camillo & Bavestrello 2008)

Figure 3.2, 3.3

Polyp: Colonies monomorphic, growing on the octocoral *Bebryce* sp. with high densities, completely covering the coenosarc of the host (Figure3.2A, B). Perisarc-free, reticular hydrorhiza growing on the surface of the octocoral host, with clusters of euryteles, and covered by a thin layer of sponge tissue (figure 3.2C-F). Gono-gastrozooids emerging from the sponge tissue (Figure 3.2C, D), up to 0.8 mm high, and with a typical pyramidal shape (Figure 3.2G). Hypostome short, surrounded by 5-7 capitate tentacles (diameter of capitula 55-65 μm). Up to 12 aboral tentacles with smaller capitula (diameter 35-45 μm). Capitula containing stenoteles of two size classes (Figure 3.2H). Basal portion of the hydranth rigid, without tentacles and with a ring of euryteles (Figure 3.2G, I-L). Hydrant highly retractile, being able to completely retract into the basal portion. Up to two medusa buds borne basally, under the sponge tissue when immature (Figure 3.2C) and outside the sponge tissue when mature. Polyp transparent with reddish gastroderm.

Medusa: Newly liberated medusa with a bell-shaped umbrella (up to 750 μm high and 600 μm wide), short manubrium, with euryteles scattered in the exumbrella (Figure 3.3A, B, G, H). Four perradial canals ending in two triangular tentaculate big bulbs and two atentaculate small bulbs. Atentaculate bulbs with an exumbrellar nematocyst pouch containing up to four euryteles (Figure 3.3C). Euryteles found also in tentaculate bulbs (Figure 3.3D). Bigger bulbs without nematocyst pouches, bearing two tentacles about 600 μm long, with up to 100 ciliated round cnidophores (diameter 25-35 μm) (Figure 3.3E) armed with 3-4 euryteles (Figure 3.3F).

Seven days old medusa with similar size and longer tentacles (up to 2 mm). Medusae transparent, with reddish bulbs and greenish manubrium.

Cnidome: i) Small stenoteles in tentacles (7-8 x 6 μm). ii) Large stenoteles in tentacles (13-14 x 9-10 μm). iii) Apotrichous macrobasic euryteles in a ring at the base of polyps, in clusters in hydrorhiza, in exumbrellar pouches, in large bulbs (26-28 x 18-20 μm; discharged shaft: 350 μm). iv) Bean-shaped apotrichous macrobasic euryteles in cnidophores (7 x 4 μm; discharged shaft: 55 μm). v) Microbasic euryteles on exumbrella (12-13 x 8-10 μm; discharged shaft: 20 μm).

Etymology: The generic name refer to the fact that the polyp stage is very similar to the *Zanclea* morphology and that the type species was previously ascribed to that genus.

Remarks: Puce et al. (2008b) described this species from Indonesia based on polyp morphology only. This species was assigned to the genus *Zanclea*, due to the shape of the polyp. Here, the medusa stage is described for the first time. The medusa is very similar to the medusa of *Pteroclava*. Notably, these two species share the apomorphy represented by the exumbrellar pouches with euryteles. However, due to the differences in the polyp stage, the new genus *Pseudozanclea* is here erected. Few differences are present between Maldivian and Indonesian specimens. In particular, Puce et al. (2008b) described the large nematocysts at the base of hydranths as mastigophores, whereas in the present work the nematocysts can be clearly classified as euryteles. However, according to the pictures included in the original description (Puce et al. 2008b), it is not possible to see if the shaft widens distally or not. Another difference is represented by the host on which the hydrozoan has been reported, since Indonesian specimens were found on *Paratelesto* sp. while Maldivian colonies grow on *Bebryce* sp.

Genus *Pteroclava* Weill 1931

Pteroclava krempfi Billard 1919

Figure 3.4, 3.5

Polyp: Colonies monomorphic, living in association with several octocorals (Figure 3.4A-H). Hydrorhiza embedded in host tissues and covered by perisarc. Gastrozooids claviform up to 1.2 mm high, with four oral and up to 18 scattered aboral moniliform or quasi-moniliform tentacles with stenoteles of two size classes (Figure 3.4I, M). Perisarc-covered pedicels reaching a maximum length of 300 μm and mostly sunken in host tissues. Up to two rounded patches of euryteles below the oral tentacles (Figure 3.4J, N, O). Up to three medusa buds carried on short pedicels on gono-gastrozooids (Figure 3.4K). In some cases, gono-gastrozooid degenerated, without tentacles (reproductive exhaustion) (Figure 3.4L). Polyps transparent.

Medusa: Newly liberated medusa with a bell-shaped umbrella (up to 800 μm high and 850 μm wide), with euryteles scattered on the exumbrella (Figure 3.5A, B, G, H). Four perradial canals ending in two tentaculate large bulbs (diameter up to 150 μm) and two atentaculate small bulbs (diameter up to 50 μm). Atentaculate bulbs with an exumbrellar nematocyst pouch containing up to four euryteles (Figure 3.5C). Euryteles often found also in tentaculate bulbs (Figure 3.5D). Large bulbs without nematocyst pouches, bearing two tentacles

about 1 mm long, with up to 40 ciliated round cnidophores (diameter up to 25 μm) (Figure 3.5E) armed with 4-7 euryteles (Figure 3.5F). Medusae transparent.

Cnidome: i) Large stenoteles on tentacles (9-11 x 7-9 μm). ii) Small stenoteles on tentacles (5-6 x 5-4 μm) iii) Apotrichous macrobasic euryteles in hydranths, exumbrellar pouches, tentaculate bulbs (35-38 x 14-17 μm). iv) Bean-shaped apotrichous macrobasic euryteles in cnidophores (8 x 5 μm). v) Microbasic euryteles on exumbrella (7-8 x 5-6 μm; discharged shaft: 20 μm).

Remarks: All the analysed specimens were morphologically identical in both the polyp and medusa stages. No clear differences were found among colonies growing on different hosts, similarly to what was noted by Boero et al. (1995).

3.4.2. Molecular Analyses

The total genomic DNA of the ethanol-fixed samples was successfully extracted from 70 colonies and seven molecular markers were amplified for a total number of 455 sequences (*16S*: n = 70; *COX1*: n = 61; *COX3*: n = 66; *18S*: n = 63; *28S*: n = 66; *ITS*: n = 64; *H3* = 65). The total alignments of the *16S*, *COX1*, *COX3*, *18S*, *28S*, *ITS* and *H3* datasets were 565, 629, 568, 1680, 1608, 536, and 342 bp long, respectively, and the concatenated dataset was 5928 bp long. PartitionFinder found the following partition scheme and models for the concatenated dataset (AIC): *16S COX3*_pos1 (GTR+ G + I), *COX1*_pos1 (SYM + G + I), *COX1*_pos2 *COX3*_pos2 (HKY + G + I), *COX1*_pos3 (HKY + G + I), *COX3*_pos3 (HKY + G), *18S* (GTR + G + I), *28S* (K80 + I), *ITS* (SYM + G), *H3*_pos1 (HKY + G), *H3*_pos2 (SYM + I), *H3*_pos3 (JC). Phylogenetic trees obtained from BI and ML analyses were identical and only the Bayesian topology with full branch supports (Bayesian posterior probability (BPP) > 0.95 and maximum likelihood bootstrapping (BS) > 95) indicated by asterisks is shown in Figure 3.6. The single- and multi-locus phylogenetic analyses gave similar results, and only the tree resulting from the concatenated analysis is shown, representing the best supported phylogenetic hypothesis.

Cladocorynidae were rooted with representative of the families Asyncorynidae Kramp 1949, Hydrocorynidae Rees 1957, Milleporidae Fleming 1828, Moerisiidae Poche 1914, Pennariidae McCrady 1859, Porpitidae Goldfuss 1818, Solanderiidae Marshall 1892, Sphaerocorynidae Prévot 1959, and Zancleidae Russel 1953, resulting in a fully supported monophyletic clade. Overall, the statistical support for the obtained relationships is very high and most of the nodes have maximal BPP and BS values. The three genera *Cladocoryne*, *Pteroclava* and *Pseudozanclea* are confirmed to constitute all together the family Cladocorynidae. In particular *Pteroclava* and *Pseudozanclea* are sister clades, whereas *Cladocoryne* is sister of the clade *Pteroclava* + *Pseudozanclea*. The two *Cladocoryne* species included in the analyses (i.e. *Cladocoryne haddoni* and *Cladocoryne floccosa*) are reciprocally monophyletic and well separated from each other. *Pteroclava krempfi* shows a high genetic diversity and can be subdivided in four major lineages (*P. krempfi* I-IV). Clade I is composed of hydrozoans associated with four genera of octocorals in the family Alcyoniidae, which are *Rhytisma* from the Red Sea and *Lobophytum*, *Sarcophyton*, and *Sinularia* from the Maldives and the Red Sea, and shows a within-clade moderate genetic structuring. Clade II is associated with *Paraplexaura* living at 40

m deep in the Maldives and with *Astrogorgia* and *Melithaea* inhabiting shallow waters in the central Red Sea. Clade III is found exclusively in the Caribbean Sea and associated with *Antillogorgia*. Finally, Clade IV includes samples from Indonesia and living on the alcyoniid genus *Sinularia*.

Within-clade genetic diversity is variable in the clades (Table 3.1). *Cladocoryne floccosa* shows a very high intra-specific mean genetic distance, especially for mitochondrial markers (*16S*: 6.5 %, *COX3*: 6 %), whereas *C. haddoni* and *Pseudozanclea timida* have low to moderate levels of intra-specific divergence. Regarding the *P. krempfi* lineages, clades I and IV have higher level of intra-clade divergence than Clade II and III. Contrarily, inter-clade genetic distances are remarkably high for all comparisons (Table 3.1). Among the mitochondrial markers used in this study, *16S* is the less variable but it nevertheless shows extremely high genetic distances even for closely related lineages. For instance, distances among *P. krempfi* lineages span from about 7.5 % to more than 13 %. Also *16S* genetic divergence between the two *Cladocoryne* species is very high, with a distance of almost 12 %. Other mitochondrial markers largely exceed these values, with the highest genetic distances recorded among *COX3* sequences. Regarding nuclear markers, high distances are found in the *ITS* and *H3* datasets, whereas *18S* and *28S* sequences show lower values.

Species delimitation analyses revealed that *P. krempfi* is a complex of cryptic species. All analyses were congruent for single- and multi-locus datasets and the rfour main lineages are ascribable to independent molecular species (Figure 3.7). In some cases, some of the methods recovered a further subdivision of the clades, but only the clusters recovered in all the analyses are here considered as independent species.

CAOS software found several specific diagnostic positions in the molecular markers (Table 3.2) and each molecular lineage was named following the molecular nomenclature proposed by Morard et al. (2016): Clade I is *P. krempfi* I, Clade II is *P. krempfi* II, Clade III is *P. krempfi* III, and Clade IV is *P. krempfi* IV.

AMOVA analyses of the *16S* sequences of the populations of *P. krempfi* I and *P. krempfi* II living in the Maldives and Red Sea showed that the intra-specific genetic diversification is mostly explained by the geographic provenience (71.09 % in *P. krempfi* I and 72.21 % in *P. krempfi* II).

3.5. DISCUSSION

The family Cladocorynidae was initially erected to accommodate the genus *Cladocoryne* (Allman 1872) and only later Petersen (1990) and Boero et al. (1995) proposed to include also the genus *Pteroclava*. This conclusion was justified by the synapomorphy represented by the presence of patches of large euryteles in the polyp stages of both genera. Morphological analyses of *Pseudozanclea timida* showed that, also in this species, aggregations of euryteles occur in the polyp stage, even if organised in rings other than patches, suggesting a possible relationship with the family Cladocorynidae. Moreover, these nematocysts are similar in shape and size to the smaller euryteles found at the base of proximal tentacles in *Cladocoryne haddoni*. Another apomorphy of the family is the presence of exumbrellar pouches with eurytele capsules, a peculiarity never found in other hydrozoan species. This feature was observed in *Pteroclava krempfi*, since the genus *Cladocoryne* reproduces via cryptomedusoids (Boero et al. 1995). However, similar euryteles are reported in

the exumbrella of *C. haddoni* and *Cladocoryne floccosa* (Bouillon et al. 1987, Migotto 1996). The medusa stage of *P. timida* highly resembles *P. krempfi*, both in the general shape of the medusa and for the presence of euryteles in exumbrellar pouches and bulbs, this latter feature strongly suggesting that the two species are sister taxa. Molecular phylogenetics results confirm these morphology-based hypotheses. Indeed, the family Cladocorynidae is fully supported by molecules, and falls within the superfamily Zancleida as a sister group of the clade Asyncorynidae + Milleporidae + Porpitidae + Solanderiidae + Zancleidae. *Pteroclava* and *Pseudozanclea* are sister taxa, in agreement with the similarities in the medusa stage, whereas *Cladocoryne* appears to be the earliest cladocorynid diverging group in the obtained phylogenetic hypothesis. The association with octocorals is another trait shared by the two medusa-producing species, even if they are associated to a variety of different hosts. Contrarily, *Cladocoryne* is a generalist taxon, living on several biotic and abiotic substrates.

Due to the inclusion of *Pseudozanclea* within the Cladocorynidae a new definition for the family is needed. Therefore, the diagnosis is emended as follows: 'stem simple or slightly branched, rising from a creeping stolon crawling within tissues of octocoral hosts or on biotic and abiotic substrates; polyp club-shaped, oral tentacles moniliform or capitate, in one whorl, aboral tentacles moniliform, capitate or branched capitate, scattered or in several whorls; cnidocysts on body wall arranged in conspicuous rounded patches, scattered around the base of oral and aboral tentacles or organised in a ring at the base of hydranths; gonophores carried singly or on short, branched pedicels, on lower or middle part of the hydranth; with free medusae or fixed cryptomedusoids. Medusa with two exumbrellar pouches, containing macrobasic euryteles, on non tentaculate perradial marginal bulbs; tentaculate perradial marginal bulbs large, without cnidocyst pouches but often with macrobasic euryteles; tentacles with cnidophores; gonads interradial on manubrium'.

In the phylogenetic analyses presented in this work, two species of *Cladocoryne* out of the five accepted were included. Indeed, *Cladocoryne littoralis*, *Cladocoryne travancorensis*, and *Cladocoryne minuta* were never found after their description and no ethanol-fixed material suitable for molecular analyses is available. Even if these species clearly belong to the family, it is not clear whether they could constitute different genera, and only further molecular analyses can answer this issue. However, the main difference among *Cladocoryne* species is represented by the tentacle organisation, and these variations are likely to be related to inter-specific other than inter-generic variation, contrarily to what was proposed by Mammen (1963). In fact, the two species included in the analyses (i.e. *C. floccosa* and *C. haddoni*), mainly differing in the number of whorls of tentacles, are closely related and the genetic distances between the two species are comparable to the intra-generic distances in the *P. krempfi* complex. These results support the synonymisation of *Lobocoryne* and *Cladocorynopsis* with *Cladocoryne* made by Bouillon et al. (1987). *Cladocoryne floccosa* and *C. haddoni* show high level of intra-specific divergence, as revealed by genetic distance values, especially for mitochondrial markers. Specifically, *C. floccosa* has a mean intra-specific distance of about 6.5 % for *16S rRNA* and this value is certainly high compared to other intra-specific values in cladocorynid species. A possible explanation is that the morphology of *C. floccosa* hides the presence of multiple cryptic or pseudocryptic species. However, it is not possible to establish whether the samples included in the analysis as

C. floccosa constitute a complex of species, due to the few specimens available. As suggested by Schuchert (2003), slight differences in tentacle number and arrangement may account for inter-specific variation and his doubts about the presence of undescribed species hidden under the *C. floccosa* or *C. haddoni*-like morphology may be supported by further detailed morphological analyses combined with molecular assessments.

The analysis of the morphology of Maldivian specimens of *Zanclea timida*, as well as the description of its medusa stage and the molecular phylogenetic analyses allowed the reassignment of this species to the here established new genus *Pseudozanclea* and the inclusion in the family Cladocorynidae. The species was formerly described from Indonesian waters and in association with the octocoral *Pratelesto* sp. (Puce et al. 2008b). After its description, no other records are available in literature and the finding of specimens in the Maldives and in association with *Bebryce* sp. widens both the host preference and the distributional range of the species. The Maldivian polyp stage is characterised by the presence of a basal ring of euryteles other than mastigophores, as described in the Indonesian polyps. A little doubt remains about the real nature of Indonesian heteronemes, since in the SEM picture in the original description (Puce et al. (2008b), Figure 7G) the terminal part of the shaft is not clearly visible and may also have been damaged during the sample preparation. If these nematocysts are confirmed to be mastigophores, populations from Maldives and Indonesia may actually correspond to two different species. However, since the two populations are identical for the remaining morphological characters and only further comparisons as well as the inclusion of Indonesian *P. timida* in the molecular analysis will possibly elucidate this point, specimens from both localities are here provisionally considered conspecific. The medusa stage was reared for seven days and showed no notable variation in shape and size during this period of time, except for the elongation of tentacles. The newly-liberated medusa is extremely similar to *P. krempfi* medusa and this explains the close relationships between the two taxa, despite the differences in the polyp stage, having *P. timida* a more ancestral *Zanclea*-like bauplan and *P. krempfi* a different type of hydranth with moniliform tentacles. Interestingly, all the colonies of *Bebryce* sp. sampled during this study hosted *P. timida* and, in all cases, a third organism was recorded in association with the octocoral and hydrozoan. Indeed, an orange unidentified sponge was constantly found covering the octocoral and hydrozoan coenosarc. Medusa buds were often found under this thin layer of tissue when immature, but were nonetheless observed free from the poriferan tissues when ready for the release. The potential beneficial or negative outputs for each organism involved in this association are still not clear but the presence of the associates seems not to inhibit the ability of reproduce, at least for the hydrozoan.

Another trait shared by *P. timida* and *P. krempfi* is the association with octocorals. With this work, the host range of the *P. krempfi* species complex is updated, since it was found for the first time in association with the genera *Sarcophyton, Lobophytum, Sinularia, Rhytisma, Paraplexaura, Antillogorgia,* and *Melithaea*, and with the families Gorgoniidae and Melithaeidae. Also the known distribution range is here extended, with new records from the Red Sea and Maldives. According to morphology, all the specimens that were analysed were identified as *P. krempfi*. The other described *Pteroclava* species, *Pteroclava crassa*, is so far identifiable only through its hydrozoan host (*Macrorhynchia philippina*), because its hydrants are almost identical to those of *P. krempfi*. Moreover, no information is available about its medusa. Boero et al. (1995) maintained the two

species separate, leaving nevertheless doubts about their conspecificity. All our specimens showed typical *Pteroclava* features, such as a perisarc-covered pedicel, moniliform tentacles, euryteles patches and exumbrellar euryteles pouches; in addition, they were collected from octocoral hosts, supporting the conclusion that they belong to the species *P. krempfi*. Contrarily to what is inferable from their morphology, a remarkable genetic differentiation was found among specimens collected from different hosts and localities. The phylogenetic reconstruction revealed the presence of four main lineages, one growing on Alcyoniidae (*Sarcophyton*, *Lobophytum*, *Sinularia*, *Rhytisma*) from Maldives and Red Sea, one growing on Plexauridae (*Paraplexaura*, *Astrogorgia*) and Melithaeidae (*Melithaea*) from Maldives and Red Sea, one growing on Gorgoniidae (*Antillogorgia*) from the Caribbean, and another one growing on Alcyoniidae (*Sinularia*) from Indonesia. Molecular data left no doubt about the strong genetic separation between these clades, since the phylogenetic analyses showed a strongly supported subdivision into four monophyletic and highly divergent lineages. Also the mean genetic distances among these groups are very high, for instance largely exceeding the *16S rRNA* intraspecific threshold values found for other hydrozoan taxa (Moura et al. 2008, Folino-Rorem et al. 2009, Miglietta et al. 2009, Moura et al. 2011, Moura et al. 2012a, Moura et al. 2012b, Zheng et al. 2014). Moreover, species delimitation analyses confirmed the presence of four cryptic species corresponding to the four main lineages. Crypticism is a common phenomenon in hydrozoans, especially for those species with few distinctive morphological features (e.g. Miglietta et al. (2009)). In the last 10 years, molecular techniques have allowed taxonomists to discover the hidden genetic diversity of several hydrozoan taxa (Govindarajan et al. 2005, Folino-Rorem et al. 2009, Lindner et al. 2011, Schuchert 2014) and this cryptic diversity could sometimes be ascribed to specific factors, such as depth, geographic distribution and hosts (Govindarajan et al. 2005, Moura et al. 2008, Montano et al. 2015). According to genetic evidence, *P. krempfi* represents a species complex, with the main diagnostic feature, other than the DNA sequences, being the host specificity and geographic provenience. Specifically, host-specificity and geographic distribution alone do not allow the distinction of the four species, but the combination of both can be used to discriminate different species: the species corresponding to Clade I and IV are associated with Alcyoniidae, with the first one found in Maldives and Red Sea and the second one in Indonesia; the species represented by Clade II lives in Maldives and Red Sea on Plexauridae and Melithaeidae; and the species corresponding to Clade III is associated with Gorgoniidae in the Caribbean. No molecular data are so far available for the originally described *P. krempfi*, which was associated with the soft coral *Cladiella krempfi* (Hickson 1919) (Billard 1919). This hydroid could be part of one of the herein identified molecular clades or it could represent another cryptic species. Moreover, given the possibility that one of the main difference among other *Pteroclava* species is the host, it is most likely that *P. crassa* is an independent species. Finally, some of the species herein identified show a further genetic structure, as shown by population genetics analyses. In particular, the intra-specific diversity of *P. krempfi* I and II is mostly explained by the geographic provenience of each colony, with significant and high *Fst* values for populations from Red Sea and Maldives of both species.

Recently, several hydroids belonging to the genus *Zanclea* Gegenbaur 1856 were discovered to grow on scleractinian corals (Boero et al. 2000, Pantos and Bythell 2010, Hirose and Hirose 2011, Montano et al. 2013,

Montano et al. 2014). Molecular analyses demonstrated that some of these hydrozoans that appeared morphologically similar or even identical were actually genetically highly divergent (Maggioni et al. In preparation). This genetic structure could be partially explained by the host specificity of different lineages, similarly to *Pteroclava*, where cryptic species grow on different octocorals with various levels of host fidelity. In a similar way, parasitic snails (*Leptoconchus* spp.) living inside scleractinian corals also show little morphological variation but a genetic differentiation at the species level that reflects the associations with their various coral hosts (Gittenberger et al. 2011). A possible explanation of these sympatric cryptic speciations might be the host shift and the subsequent isolation of a symbiont population. A role for bacteria in larval settlement and subsequent metamorphosis into primary polyp has been demonstrated in different hydroid species (Müller 1969, Edwards et al. 1987, Thomas et al. 1987, Freeman and Ridgway 1990). Changes in planula receptors of *P. krempfi* might have caused a different response to host-associated microorganisms and might have led to a shift from an octocorals to another. Conversely, variations in the host holobiome might have promoted this shift. These events could have played a role alone or in conjunction with other factors, such as different optimal depths of the hosts and, in some cases, isolation by distance, and need to be tested with future studies.

The clarification of the systematics of the *Pteroclava krempfi* species complex, as well as the ecology of the symbiosis in which it is involved, represent important steps in the understanding of the ecological and taxonomical complexity of coral reef ecosystems, especially during the current drastic changes they are facing (Hughes et al. 2017). Indeed, despite alcyonacean octocorals are abundant and ecologically important members of coral reef communities, often equalling or exceeding scleractinian corals in their percent cover of available primary space (Stobart et al. 2005), few studies have examined the diversity and community structure of the invertebrates that dwell on octocorals. For instance, recently Montano et al. (2016) investigated the ecology of *P. krempfi* I, showing that the association with Alcyoniidae was common in the explored areas and that the hydrozoan may have preferences regarding the reef type and the genus of the host. Since the outputs of these symbiotic associations are still unknown for both symbionts, similarly to scleractinian-associated hydrozoans, further studies need to be urgently carried out in order to characterise these poorly understood, but intimate relationships that often go unrecognized and underestimated in their importance.

3.6. REFERENCES

Allman GJ (1872) *A Monograph of the Gymnoblastic or Tubularian Hydroids*. Robert Hardwicke.

Appeltans W, Ahyong ST, Anderson G, Angel MV, Artois T, Bailly N et al., Costello MJ (2012) The magnitude of global marine species diversity. *Current Biology* 22: 2189-2202.

Bergmann T, Hadrys H, Breves G, Schierwater B (2009) Character-based DNA barcoding: a superior tool for species classification. *Berliner und Munchener Tierarztliche Wochenschrift* 122: 446-450.

Bickford D, Lohman DJ, Sodhi NS, Ng PK, Meier R, Winker K et al., Das I (2007) Cryptic species as a window on diversity and conservation. *Trends in Ecology & Evolution* 22: 148-155.

Billard A (1919) Note sur une espèce nouvelle d'hydroïde gymnoblastique (*Clava krempfi*), parasite d'un Alcyonaire. *Bulletin du Muséum national d'Histoire naturelle de Paris* 25: 187-188.

Bo M, Di Camillo CG, Puce S, Canese S, Giusti M, Angiolillo M, Bavestrello G (2011) A tubulariid hydroid associated with anthozoan corals in the Mediterranean Sea. *Italian Journal of Zoology* 78: 487-496.

Boero F, Bouillon J, Gravier-Bonnet N (1995) The life cycle of *Pteroclava krempfi* (Cnidaria, Hydrozoa, Cladocorynidae), with notes on *Asyncoryne philippina* (Asyncorynidae). *Scientia Marina(Barcelona)* 59: 65-76.

Boero F, Bouillon J, Gravili C (2000) A survey of *Zanclea, Halocoryne* and *Zanclella* (Cnidaria, Hydrozoa, Anthomedusae, Zancleidae) with description of new species. *Italian Journal of Zoology* 67: 93-124.

Bouillon J, Boero F, Seghers G (1987) Redescription of *Cladocoryne haddoni* Kirkpatrick and a proposed phylogeny of the superfamily Zancleoidea (Anthomedusae, Hydrozoa, Cnidaria). *Indo-Malayan Zoology* 4: 279-292.

Bouillon J, Gravili C, Gili J-M, Boero F (2006) *An Introduction to Hydrozoa*. Publications Scientifiques du Museum, Paris.

Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution* 17: 540-552.

Colgan D, McLauchlan A, Wilson G, Livingston S, Edgecombe G, Macaranas J et al., Gray M (1998) Histone H3 and U2 snRNA DNA sequences and arthropod molecular evolution. *Australian Journal of Zoology* 46: 419-437.

Cunningham CW, Buss LW (1993) Molecular evidence for multiple episodes of paedomorphosis in the family Hydractiniidae. *Biochemical Systematics and Ecology* 21: 57-69.

Drummond A, Ashton B, Buxton S, Cheung M, Cooper A, Heled J et al., Sturrock S (2010) Geneious v6. 1.6. *Website: http://www. geneious. com*.

Drummond AJ, Suchard MA, Xie D, Rambaut A (2012) Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution* 29: 1969-1973.

Edwards NC, Thomas MB, Long BA, Amyotte SJ (1987) Catecholamines induce metamorphosis in the hydrozoan *Halocordyle disticha* but not in *Hydractinia echinata*. *Development Genes and Evolution* 196: 381-384.

Excoffier L, Lischer HE (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* 10: 564-567.

Ezard T, Fujisawa T, Barraclough T (2009) Splits: species' limits by threshold statistics. *R package version* 1.

Fabricius KK, Alderslade PP (2001) *Soft Corals and Sea Fans: a Comprehensive Guide to the Tropical Shallow Water Genera of the Central-West Pacific, the Indian Ocean and the Red Sea*. Australian Institute of Marine Science.

Fisher R, O'Leary RA, Low-Choy S, Mengersen K, Knowlton N, Brainard RE, Caley MJ (2015) Species richness on coral reefs and the pursuit of convergent global estimates. *Current Biology* 25: 500-505.

Folino-Rorem NC, Darling JA, D'Ausilio CA (2009) Genetic analysis reveals multiple cryptic invasive species of the hydrozoan genus *Cordylophora*. *Biological Invasions* 11: 1869-1882.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* 3: 294-299.

Fontana S, Keshavmurthy S, Hsieh HJ, Denis V, Kuo C-Y, Hsu C-M et al., Chen CA (2012) Molecular evidence shows low species diversity of coral-associated hydroids in *Acropora* corals. *PloS One* 7: e50130.

Freeman G, Ridgway EB (1990) Cellular and intracellular pathways mediating the metamorphic stimulus in hydrozoan planulae. *Development Genes and Evolution* 199: 63-79.

Gili J-M, López-González PJ, Bouillon J (2006) A new Antarctic association: the case of the hydroid *Sarsia medelae* (new sp.) associated with gorgonians. *Polar Biology* 29: 624-631.

Gittenberger A, Reijnen BT, Hoeksema BW (2011) A molecularly based phylogeny reconstruction of mushroom corals (Scleractinia Fungiidae) with taxonomic consequences and evolutionary implications for life history traits. *Contributions to Zoology* 80: 107-132.

Goh NK, Ng PK, Chou L (1999) Notes on the shallow water gorgonian-associated fauna on coral reefs in Singapore. *Bulletin of Marine Science* 65: 259-282.

Govindarajan AF, Halanych KM, Cunningham CW (2005) Mitochondrial evolution and phylogeography in the hydrozoan *Obelia geniculata* (Cnidaria). *Marine Biology* 146: 213-222.

Hirohito ES (1988) *The Hydroids of Sagami Bay*. Publications of the Biolological Laboratory, Imperial Household, Tokyo.

Hirose M, Hirose E (2011) A new species of *Zanclea* (Cnidaria: Hydrozoa) associated with scleractinian corals from Okinawa, Japan. *Journal of the Marine Biological Association of the United Kingdom* 92: 877-884.

Hoeksema BW, Van der Meij SET, Fransen CHJM (2012) The mushroom coral as a habitat. *Journal of the Marine Biological Association of the United Kingdom* 92: 647-663.

Hughes TP, Kerry JT, Álvarez-Noriega M, Álvarez-Romero JG, Anderson KD, Baird AH et al., Berkelmans R (2017) Global warming and recurrent mass bleaching of corals. *Nature* 543: 373-377.

Jörger KM, Schrödl M (2013) How to describe a cryptic species? Practical challenges of molecular taxonomy. *Frontiers in Zoology* 10: 59.

Jörger KM, Schrödl M (2014) How to use CAOS software for taxonomy? A quick guide to extract diagnostic nucleotides or amino acids for species descriptions. *Spixiana* 37: 21-26.

Katoh K, Standley DM (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution* 30: 772-780.

Kirkpatrick R (1890). Reports on the zoological collections made in Torres Straits by Professor AC Haddon, 1888-1889. Hydroida and Polyzoa. *Scientific Proceedings of the Royal Dublin Society, New Series* 6: 603-626.

Knowlton N (2000) Molecular genetic analyses of species boundaries in the sea. *Hydrobiologia* 420: 73-90.

Lanfear R, Calcott B, Ho SY, Guindon S (2012) PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution* 29: 1695-1701.

Lindner A, Govindarajan AF, Migotto AE (2011) Cryptic species, life cycles, and the phylogeny of *Clytia* (Cnidaria: Hydrozoa: Campanulariidae). *Zootaxa* 2980: 23-36.

Love MS, Yoklavich MM, Black BA, Andrews AH (2007) Age of black coral (*Antipathes dendrochristos*) colonies, with notes on associated invertebrate species. *Bulletin of Marine Science* 80: 391-399.

Maggioni D, Montano S, Seveso D, Galli P (2016) Molecular evidence for cryptic species in *Pteroclava krempfi* (Hydrozoa, Cladocorynidae) living in association with alcyonaceans. *Systematics and Biodiversity* 14: 484-493.

Mammen T (1963) On a collection of hydroids from South India. I. Suborder Athecata. *Journal of the Marine Biological Association of India* 5: 27-61.

Medlin L, Elwood HJ, Stickel S, Sogin ML (1988) The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene* 71: 491-499.

Miglietta MP, Schuchert P, Cunningham CW (2009) Reconciling genealogical and morphological species in a worldwide study of the Family Hydractiniidae (Cnidaria, Hydrozoa). *Zoologica Scripta* 38: 403-430.

Migotto AE (1996) Benthic shallow-water hydroids (Cnidaria, Hydrozoa) of the coast of São Sebastião, Brazil, including a checklist of Brazilian hydroids. *Zoologische Verhandelingen* 306: 1-125.

Monaghan MT, Wild R, Elliot M, Fujisawa T, Balke M, Inward DJ et al., Vogler AP (2009) Accelerated species inventory on Madagascar using coalescent-based models of species delineation. *Systematic Biology* 58: 298-311.

Montano S, Allevi V, Seveso D, Maggioni D, Galli P (2016) Habitat preferences of the *Pteroclava krempfi*-alcyonaceans symbiosis: inner vs outer coral reefs. *Symbiosis* 72: 225-231.

Montano S, Galli P, Maggioni D, Seveso D, Puce S (2014) First record of coral-associated *Zanclea* (Hydrozoa, Zancleidae) from the Red Sea. *Marine Biodiversity* 44: 581-584.

Montano S, Maggioni D, Arrigoni R, Seveso D, Puce S, Galli P (2015) The Hidden Diversity of *Zanclea* Associated with Scleractinians Revealed by Molecular Data. *PloS One* 10: e0133084.

Montano S, Maggioni D, Galli P, Seveso D, Puce S (2013) *Zanclea*-coral association new records from Maldives. *Coral Reefs* 32: 701.

Morard R, Escarguel G, Weiner AK, André A, Douady CJ, Wade CM et al., Quillévéré F (2016) Nomenclature for the nameless: a proposal for an integrative molecular taxonomy of cryptic diversity exemplified by planktonic foraminifera. *Systematic Biology* 65: 925-940.

Moura CJ, Cunha MR, Porteiro FM, Rogers AD (2011) The use of the DNA barcode gene 16S mRNA for the clarification of taxonomic problems within the family Sertulariidae (Cnidaria, Hydrozoa). *Zoologica Scripta* 40: 520-537.

Moura CJ, Cunha MR, Porteiro FM, Rogers AD (2012a) Polyphyly and cryptic diversity in the hydrozoan families Lafoeidae and Hebellidae (Cnidaria: Hydrozoa). *Invertebrate Systematics* 25: 454-470.

Moura CJ, Cunha MR, Porteiro FM, Yesson C, Rogers AD (2012b) Evolution of *Nemertesia* hydroids (Cnidaria: Hydrozoa, Plumulariidae) from the shallow and deep waters of the NE Atlantic and western Mediterranean. *Zoologica Scripta* 41: 79-96.

Moura CJ, Harris DJ, Cunha MR, Rogers AD (2008) DNA barcoding reveals cryptic diversity in marine hydroids (Cnidaria, Hydrozoa) from coastal and deep-sea environments. *Zoologica Scripta* 37: 93-108.

Müller W (1969) Auslösung der Metamorphose durch Bakterien bei den Larven von *Hydractinia echinata*. *Zoologische Jahrbücher* 86: 84-95.

Nagale P, Apte D (2014) Intertidal hydroids (Cnidaria: Hydrozoa: Hydroidolina) from the Gulf of Kutch, Gujarat, India. *Marine Biodiversity Records* 7: e116.

Pantos O, Bythell JC (2010) A novel reef coral symbiosis. *Coral Reefs* 29: 761-770.

Paradis E, Strimmer K, Claude J, Jobb G, Opgen-Rhein R, Dutheil J et al., Lemon J (2004) ape: Analyses of Phylogenetics and Evolution. R package version 1.3-1. *Comprehensive R Archive Network, Vienna, Austria*.

Patton WK (1972) Studies on the animal symbionts of the gorgonian coral, *Leptogorgia virgulata* (Lamarck). *Bulletin of Marine Science* 22: 419-431.

Peña-Cantero ÁL, Sentandreu V (2017) Phylogenetic relationships of endemic Antarctic species of *Staurotheca* Allman, 1888 (Cnidaria, Hydrozoa). *Polar Biology* 40: 301-312.

Petersen KW (1990) Evolution and taxonomy in capitate hydroids and medusae (Cnidaria: Hydrozoa). *Zoological Journal of the Linnean Society* 100: 101-231.

Pictet C (1893) Étude sur les hydraires de la Baie d'Amboine. *Revue Suisse de Zoologie* 1: 1-64.

Pons J, Barraclough TG, Gomez-Zurita J, Cardoso A, Duran DP, Hazell S et al., Vogler AP (2006) Sequence-based species delimitation for the DNA taxonomy of undescribed insects. *Systematic Biology* 55: 595-609.

Puce S, Cerrano C, Di Camillo CG, Bavestrello G (2008a) Hydroidomedusae (Cnidaria: Hydrozoa) symbiotic radiation. *Journal of the Marine Biological Association of the United Kingdom* 88: 1715-1721.

Puce S, Di Camillo CG, Bavestrello G (2008b) Hydroids symbiotic with octocorals from the Sulawesi Sea, Indonesia. *Journal of the Marine Biological Association of the United Kingdom* 88: 1643-1654.

Puillandre N, Lambert A, Brouillet S, Achaz G (2012) ABGD, Automatic Barcode Gap Discovery for primary species delimitation. *Molecular Ecology* 21: 1864-1877.

Rambaut A, Drummond A (2013) TreeAnnotator v1. 7.0. *Available as part of the BEAST package at http://beast. bio. ed. ac. uk.*

Rambaut A, Suchard M, Xie W, Drummond A (2014) Tracer v. 1.6. Institute of Evolutionary Biology, University of Edinburgh.

Reaka-Kudla ML, Wilson DE, Wilson EO (1996) *Biodiversity II: Understanding and Protecting Our Biological Resources*. Joseph Henry Press.

Reid NM, Carstens BC (2012) Phylogenetic estimation error can decrease the accuracy of species delimitation: a Bayesian implementation of the general mixed Yule-coalescent model. *BMC Evolutionary Biology* 12: 196.

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S et al., Huelsenbeck JP (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* 61: 539-542.

Ruppert EEB, Fox RD, Ruppert RSEE, Fox RS, Barnes RD (2004) *Invertebrate Zoology: a Functional Evolutionary Approach*. Brooks-Cole Publishing.

Sarkar IN, Planet PJ, Desalle R (2008) CAOS software for use in character-based DNA barcoding. *Molecular Ecology Resources* 8: 1256-1259.

Sarkar IN, Thornton JW, Planet PJ, Figurski DH, Schierwater B, DeSalle R (2002) An automated phylogenetic key for classifying homeoboxes. *Molecular Phylogenetics and Evolution* 24: 388-399.

Schuchert P (2003) Hydroids (Cnidaria, Hydrozoa) of the Danish expedition to the Kei Islands. *Steenstrupia* 27: 137-256.

Schuchert P (2014) High genetic diversity in the hydroid *Plumularia setacea*: a multitude of cryptic species or extensive population subdivision? *Molecular Phylogenetics and Evolution* 76: 1-9.

Stella JS, Pratchett MS, Hutchings PA, Jones GP (2011) Coral-associated invertebrates: diversity, ecological importance and vulnerability to disturbance. *Oceanography and Marine Biology: an Annual Review* 49: 43-104.

Sukumaran J, Holder MT (2010) DendroPy: a Python library for phylogenetic computing. *Bioinformatics* 26: 1569-1571.

Talavera G, Castresana J (2007) Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Systematic Biology* 56: 564-577.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution* 30: 2725-2729.

Thomas MB, Freeman G, Martin VJ (1987) The embryonic origin of neurosensory cells and the role of nerve cells in metamorphosis in *Phialidium gregarium* (Cnidaria, Hydrozoa). *International Journal of Invertebrate Reproduction and Development* 11: 265-285.

Varela C (2010) Tres nuevos registros de hidrozoos (Cnidaria: Hydroidomedusae), para Cuba. *Revista de Investigaciones Marinas* 31: 104-105.

Watson J (2005) Hydroids of the Archipelago of the Recherche and Esperance, Western Australia: annotated list, redescription of species and description of new species. *The Marine Flora and Fauna of Esperance, Western Australia. Western Australian Museum*: 495-612.

Weill R (1931) Le genre *Pteroclava* n. gen., l'interprétation systématique des Ptéronemidae (Hydraires) et la valeur taxonomique du cnidome. *Comptes rendus hebdomadaires des Séances de l'Académie des Sciences, Paris* 192: 60-62.

Williams GC, Chen J-Y (2012) Resurrection of the octocorallian genus *Antillogorgia* for Caribbean species previously assigned to *Pseudopterogorgia*, and a taxonomic assessment of the relationship of these genera with *Leptogorgia* (Cnidaria, Anthozoa, Gorgoniidae). *Zootaxa* 3505: 39-52.

Zhang J, Kapli P, Pavlidis P, Stamatakis A (2013) A general species delimitation method with applications to phylogenetic placements. *Bioinformatics* 29: 2869-2876.

Zheng L, He J, Lin Y, Cao W, Zhang W (2014) 16S rRNA is a better choice than COI for DNA barcoding hydrozoans in the coastal waters of China. *Acta Oceanologica Sinica* 33: 55-76.

Zietara MS, Arndt A, Geets A, Hellemans B, Volckaert FA (2000) The nuclear rDNA region of *Gyrodactylus arcuatus* and *G. branchicus* (Monogenea: Gyrodactylidae). *Journal of Parasitology* 86: 1368-1373.

Zwickl D (2006) GARLI: genetic algorithm for rapid likelihood inference. *See http://www. http://www.bio.utexas.edu/faculty/antisense/garli/Garli.html*.

3.7. TABLES

Table 3.1. Genetic distances (% uncorrected p-distances) of the A) *16S*, B) *COX1*, C) *COX3*, E) *18S*, E) *28S*, F) *ITS*, G) *H3* datasets among and within (diagonal) Cladocorynidae species. Values are mean ± standard deviation.

A) *16S*	1	2	3	4	5	6	7
1) *C. floccosa*							
2) *C. haddoni*	11,8 ± 1,2	1,1 ± 0,3					
3) *P. timida*	14,3 ± 1,3	15,3 ± 1,4	0,2 ± 0,2				
4) *P. krempfi* I	13,9 ± 1,2	14,7 ± 1,5	14,8 ± 1,4	1,8 ± 0,3			
5) *P. krempfi* II	12,5 ± 1,2	12,7 ± 1,3	13,6 ± 1,4	7,3 ± 1,0	0,3 ± 0,1		
6) *P. krempfi* III	15,9 ± 1,5	15,2 ± 1,7	15,5 ± 1,6	8,9 ± 1,2	9,2 ± 1,3	1,8 ± 0,6	
7) *P. krempfi* IV	16,5 ± 1,1	17,0 ± 1,4	18,0 ± 1,4	13,2 ± 1,2	13,1 ± 1,3	12,9 ± 1,4	1,9 ± 0,8

B) *COX1*	1	2	3	4	5	6
1) *C. floccosa*	n.c.					
2) *C. haddoni*	12,8 ± 1,3	2,9 ± 0,5				
3) *P. timida*	16,4 ± 1,4	18,9 ± 1,3	0,6 ± 0,2			
4) *P. krempfi* I	17,4 ± 1,4	18,5 ± 1,3	21,2 ± 1,4	3,7 ± 0,4		
5) *P. krempfi* II	15,9 ± 1,4	18,8 ± 1,4	18,2 ± 1,4	14,4 ± 1,2	1,4 ± 0,3	
6) *P. krempfi* III	18,8 ± 1,5	18,5 ± 1,4	19,0 ± 1,4	15,8 ± 1,2	17,4 ± 1,4	1,1 ± 0,4
7) *P. krempfi* IV	20,2 ± 1,5	20,8 ± 1,3	18,6 ± 1,5	18,6 ± 1,3	19,0 ± 1,4	19,9 ± 1,4	3,9 ± 1,5

C) *COX3*	1	2	3	4	5	6
1) *C. floccosa*	5,9 ± 0,6					
2) *C. haddoni*	18,5 ± 1,4	2,7 ± 0,5				
3) *P. timida*	19,7 ± 1,4	20,9 ± 1,5	0,5 ± 0,2			
4) *P. krempfi* I	21,0 ± 1,4	20,3 ± 1,4	19,3 ± 1,5	3,5 ± 0,3		
5) *P. krempfi* II	17,9 ± 1,4	20,1 ± 1,5	18,6 ± 1,6	13,5 ± 1,3	0,8 ± 0,2	
6) *P. krempfi* III	18,8 ± 1,4	21,3 ± 1,6	20,8 ± 1,6	15,6 ± 1,2	13,4 ± 1,2	3,1 ± 0,6
7) *P. krempfi* IV	20,7 ± 1,3	22,4 ± 1,5	21,6 ± 1,4	18,5 ± 1,2	16,3 ± 1,3	17,1 ± 1,3	3,9 ± 0,6

D) *18S*	1	2	3	4	5	6
1) *C. floccosa*	0,2 ± 0,1					
2) *C. haddoni*	1,8 ± 0,3	0,0 ± 0,0				
3) *P. timida*	2,0 ± 0,3	2,3 ± 0,4	0,4 ± 0,1			
4) *P. krempfi* I	2,0 ± 0,3	2,1 ± 0,3	1,1 ± 0,2	0,1 ± 0,1		
5) *P. krempfi* II	1, 9 ± 0,3	2,1 ± 0,3	0,9 ± 0,2	0,6 ± 0,1	0,2 ± 0,1	
6) *P. krempfi* III	2,1 ± 0,3	2,2 ± 0,4	1,0 ± 0,2	0,7 ± 0,2	0,6 ± 0,2	0,0 ± 0,0
7) *P. krempfi* IV	1,9 ± 0,3	2,0 ± 0,3	1,1 ± 0,2	0,7 ± 0,2	0,6 ± 0,2	0,7 ± 0,2	0,2 ± 0,1

E) *28S*		1	2	3	4	5	6
1) *C. floccosa*	0,8 ± 0,1						
2) *C. haddoni*	3,6 ± 0,4	0,0 ± 0,0					
3) *P. timida*	4,3 ± 0,5	4,3 ± 0,5	0,0 ± 0,0				
4) *P. krempfi* I	5,0 ± 0,5	4,9 ± 0,5	3,5 ± 0,4	0,0 ± 0,0			
5) *P. krempfi* II	4,7 ± 0,5	5,0 ± 0,5	3,4 ± 0,5	0,9 ± 0,2	0,0 ± 0,0		
6) *P. krempfi* III	5,0 ± 0,5	5,0 ± 0,5	3,2 ± 0,4	1,3 ± 0,3	1,3 ± 0,2	0,3 ± 0,1	
7) *P. krempfi* IV	4,9 ± 0,5	4,7 ± 0,5	3,3 ± 0,4	1,5 ± 0,3	1,6 ± 0,3	1,7 ± 0,3	0,2 ± 0,1

F) *ITS*		1	2	3	4	5	6
1) *C. floccosa*	n.c.						
2) *C. haddoni*	20,2 ± 1,8	0,2 ± 0,1					
3) *P. timida*	20,9 ± 1,8	20,1 ± 1,8	0,0 ± 0,0				
4) *P. krempfi* I	19,8 ± 1,7	22,1 ± 1,9	10,6 ± 1,3	0,1 ± 0,0			
5) *P. krempfi* II	20,3 ± 1,7	22,3 ± 1,9	11,1 ± 1,4	1,9 ± 0,6	0,1 ± 0,0		
6) *P. krempfi* III	20,7 ± 1,7	22,6 ± 1,9	10,7 ± 1,3	2,5 ± 0,5	2,2 ± 0,5	0,6 ± 0,3	
7) *P. krempfi* IV	19,3 ± 1,7	23,2 ± 1,9	10,3 ± 1,2	5,8 ± 1,0	5,9 ± 0,9	6,2 ± 0,9	0,4 ± 0,2

G) *H3*		1	2	3	4	5	6
1) *C. floccosa*	n.c.						
2) *C. haddoni*	11,1 ± 1,2	1,2 ± 0,5					
3) *P. timida*	20,1 ± 2,2	20,6 ± 2,2	1,5 ± 0,4				
4) *P. krempfi* I	16,0 ± 1,6	18,4 ± 1,8	17,5 ± 2,3	0,4 ± 0,1			
5) *P. krempfi* II	16,5 ± 1,6	19,3 ± 1,8	17,0 ± 2,3	3,0 ± 0,9	0,1 ± 0,1		
6) *P. krempfi* III	15,8 ± 1,6	18,9 ± 1,8	16,7 ± 2,2	4,2 ± 1,0	4,0 ± 1,0	n.c.	
7) *P. krempfi* IV	15,6 ± 1,6	18,7 ± 1,8	18,4 ± 2,4	4,3 ± 1,0	5,3 ± 1,2	6,6 ± 1,3	0,6 ± 0,3

Table 3.2. Diagnostic molecular characters in the A) *16S*, B) *COX1*, C) *COX3*, D) *18S*, E) *28S*, F) *ITS*, G) *H3* sequences for the *Pteroclava krempfi* cryptic species.

	Diagnostic characters with position in alignment (in reference sequence)
A) *16S* (AY787881)	
P. krempfi I	164 (213), A; 205 (254), C; 215 (264), C; 237 (286), A; 286 (335), G; 310 (369), C; 328 (377), T; 371 (420), C
P. krempfi II	9 (58), C; 21 (70), G; 220 (269), C; 232 (281), C; 280 (329), T; 281 (330), T; 302 (351), T; 326 (375), C; 382 (431), C; 385 (434), T; 437 (486), C
P. krempfi III	216 (265), C; 225 (274), C; 233 (282), T; 246 (295), T; 310 (359), G; 377 (426), A
P. krempfi IV	13 (62), T; 18 (67), G; 23 (72), T; 41 (90), C; 101 (150), T; 149 (198), G; 221 (270), G; 232 (281), G; 243 (292), C: 250 (299), A; 309 (358), C; 381 (430), T; 434 (483), A; 503 (552), A
B) *COX1* (LT158217)	
P. krempfi I	9 (27), G; 33 (51), A; 37 (55), T; 123 (141), T; 127 (145), C; 177 (195), G; 276 (294), C; 321 (339), C; 447 (465), T-C; 558 (576), C
P. krempfi II	72 (90), C; 111 (129), C; 150 (168), T; 235 (253), T; 240 (258), A; 294 (312), C; 375 (393), T; 393 (411), T; 489 (507), T; 531 (549), T
P. krempfi III	21 (39), C; 33 (51), C; 51 (69), G; 96 (124), G; 114 (132), A; 171 (189), T; 183 (201), A; 201 (219), C; 246 (264), C; 271 (289), C; 303 (321), T; 318 (336), C; 339 (357), A; 387 (405), A; 429 (447), C; 453 (471), A; 465 (483), A; 483 (501), C; 522 (540), G; 525 (543), T; 591 (609), G
P. krempfi IV	90 (108), A; 102 (120), C; 108 (126), C; 114 (132), T; 120 (138), T; 129 (147), G; 147 (165), C; 153 (171), A; 171 (189), C; 174 (192), A; 189 (207), A; 204 (222), C; 237 (255), T; 246 (264), A; 255 (273), C; 258 (276), T; 291 (309), A; 297 (315), A; 321 (339), A; 408 (426), T; 451 (469), A; 477 (495), A; 492 (510), C; 600 (618), A
C) *COX3* (KT824445)	
P. krempfi I	60 (145), T; 69 (154), G; 120 (205), G; 144 (229), C; 303 (388), G; 333 (418), G; 340 (425), G; 343 (428), G; 351 (436), A; 393 (478), A-G; 423 (508), C; 426 (511), C
P. krempfi II	33 (118), G; 87 (172), G; 90 (175), T; 123 (208), C; 147 (232), C; 174 (259), C; 192 (277), T; 288 (367), T; 330 (415), C; 351 (436), C; 411 (496), A; 468 (553), C-T
P. krempfi III	165 (250), C; 225 (310), T; 258 (343), C-T; 297 (482), C; 369 (454), G; 483 (568), T; 489 (574), G; 493 (578), G; 504 (589), T
P. krempfi IV	19 (104), T; 63 (148), G; 102 (187), A; 129 (214), A-G; 168 (253), C; 183 (268), T; 290 (375), C-T; 336 (421), T; 351 (436), G; 367 (452), T-C; 522 (607), T-C
D) *18S* (LT593888)	
P. krempfi I	1642 (1653), A; 1644 (1655), C
P. krempfi II	172 (183), T; 1645 (1656), T; 1647 (1658), C
P. krempfi III	169 (180), A; 223 (234), C; 788 (799), G; 1006 (1017), G; 1267 (1278), T
P. krempfi IV	174 (185), G-T; 439 (450), T; 1005 (1016), G

E) *28S* (LT222045)

P. krempfi I	202 (225), C; 224 (247), C; 280 (303), C; 1339 (1362), A; 1348 (1371), C
P. krempfi II	103 (126), C; 198 (221), A; 281 (304), A; 397 (420), C; 1284 (1307), G; 1341 (1364), T; 1342 (1365), C; 1346 (1369), T
P. krempfi III	190 (213), C; 282 (305), T; 441 (464), C; 445 (468), A; 1059 (1082), G; 1342 (1365), G; 1343 (1366), T; 1345 (1368), A
P. krempfi IV	12 (35), C; 49 (72), G; 55 (78), A; 87 (110), G; 120 (143), T; 126 (159), C; 209 (232), C; 275 (298), A; 276 (299), T; 417 (440), C; 1338 (1361), T; 1343 (1366), A; 1344 (1367), C; 1345 (1368), C; 1349 (1372), G

F) *ITS* (LT606997)

P. krempfi I	52 (138), G; 151 (237), -; 193 (279), T; 203 (289), A
P. krempfi II	128 (214), G; 183 (269), T; 188 (274), -; 189 (275), -; 190 (276), -; 191 (277), -; 192 (278), -; 193 (279), -; 194 (280), -
P. krempfi III	11 (97), A; 87 (173), G; 193 (279), C; 402 (488), A; 432 (518), -; 433 (519), -; 454 (540), -
P. krempfi IV	59 (145), C; 64 (150), T; 84 (170), T; 117 (203), T; 122 (208), A; 133 (219), A; 139 (225), A; 141 (227), T; 142 (228), T; 151 (237), A; 167 (253), T; 189 (275), C; 191 (277), T; 193 (279), A; 195 (281), G; 197 (283), G; 201 (287), G; 393 (479), A

G) *H3* (AY428829)

P. krempfi I	97 (86), A; 203 (192), A
P. krempfi II	118 (107), A-T; 214 (203), C; 228 (217), T; 256 (245), A-C;
P. krempfi III	94 (83), T; 118 (107), T; 181 (170), G; 219 (208), A; 235 (224), A; 256 (245), A; 265 (254), G; 325 (314), T
P. krempfi IV	130 (119), A-C; 142 (131), T; 187 (176), C; 197 (186), C; 220 (209), C; 262 (251), A; 268 (257), G; 331 (320), A-C

3.8. FIGURES

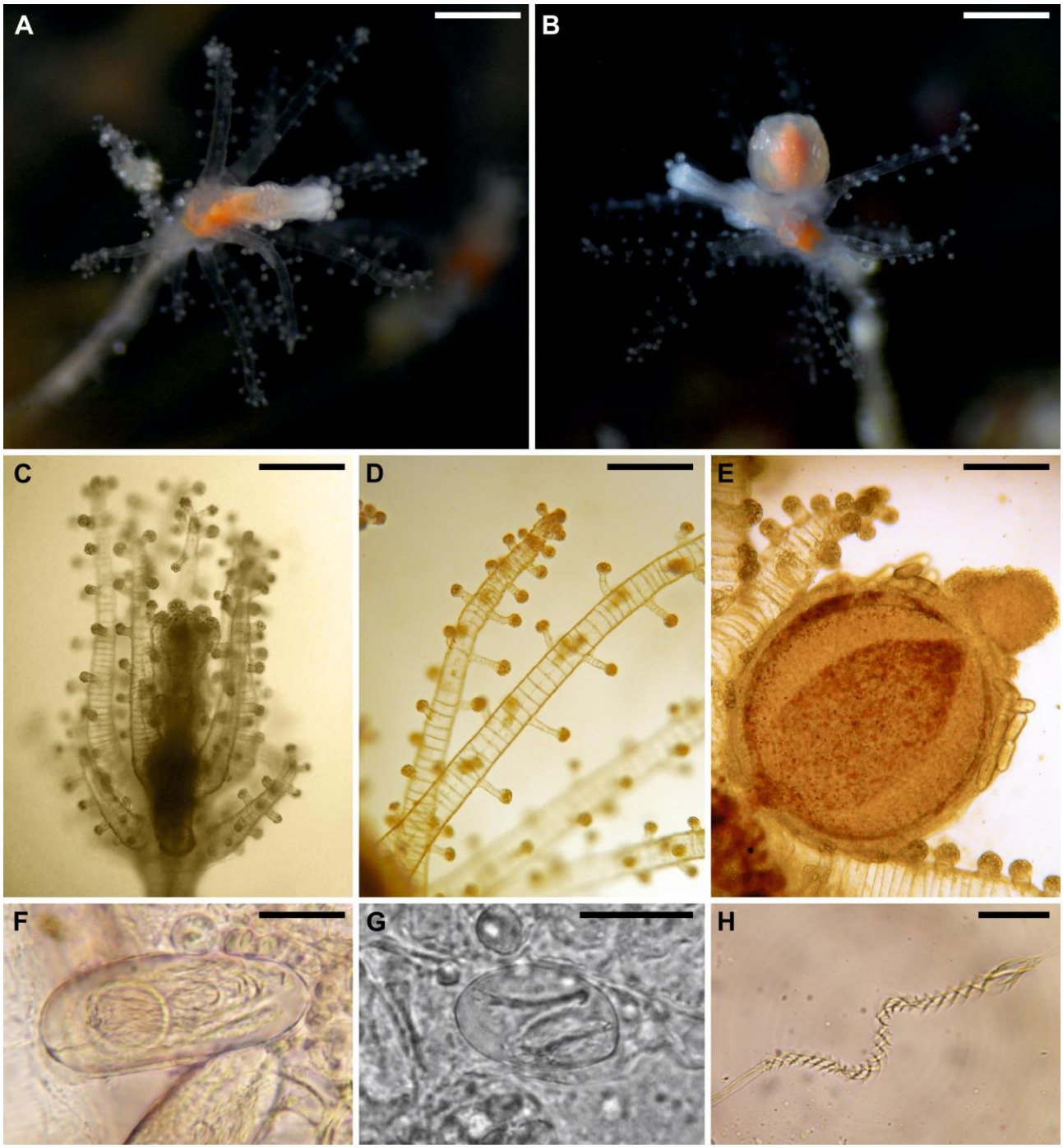

Figure 3.1. *Cladocoryne haddoni*. Micrographs of A) unfertile and B) fertile polyps of *Cladocoryne haddoni*. Light microscope micrographs of the C) polyp and details of the D) aboral ramified capitate tentacles and the E) cryptomedusoids. F) Large euryteles found in clusters and G) small euryteles at the base of tentacles, both with a H) apotrichous macrobasic shaft. Scale bars: A, B) 0.5 mm, C) 0.4 mm, D) 0.2 mm E) 120 μm, F-H) 15 μm.

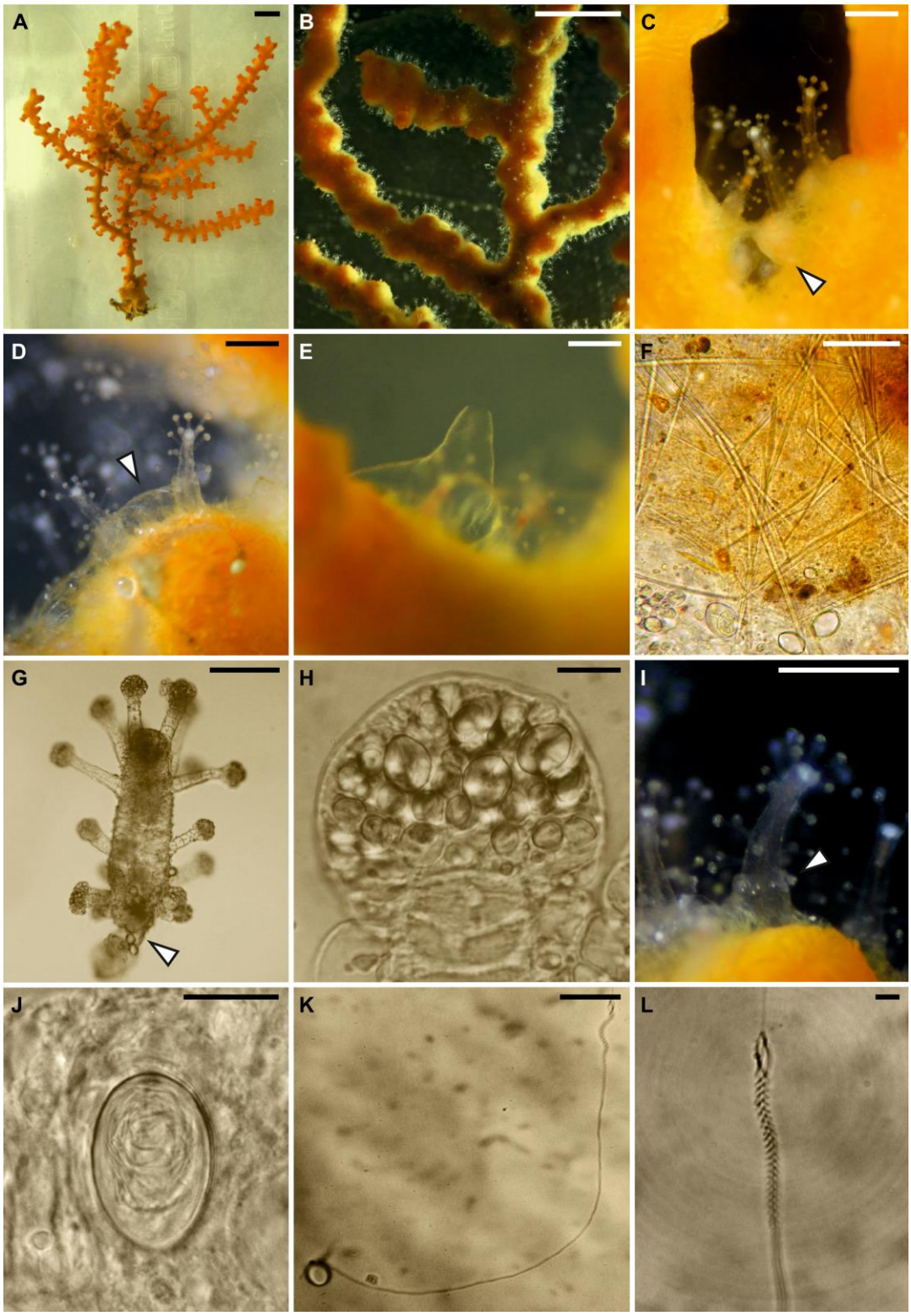

Figure 3.2. Polyp stage of *Pseudozanclea timida*. A) *Bebryce* sp. hosting *P. timida* and the unidentified sponge and a B) close-up of the association showing the high density of hydrozoan polyps. C) *P. timida* polyps with medusa buds (arrowhead) under the sponge layer. D) Two polyps covered for half of their height by the sponge (arrowhead). E, F) Osculum and spicules of the sponge, respectively. G) Light microscope micrograph of the polyp showing the presence of euryteles at the base (arrowhead). H) Large and small stenoteles contained in the capitula. I) Polyp with a band of euryteles indicated by the arrowhead. J) Undischarged capsule, K) discharged capsule and L) shaft detail of the macrobasic apotrichous euryteles. Scale bars: A, B) 2 cm, C-E, I) 0.5 mm, F, K) 50 µm, G) 250 µm, H, J, L) 15 µm.

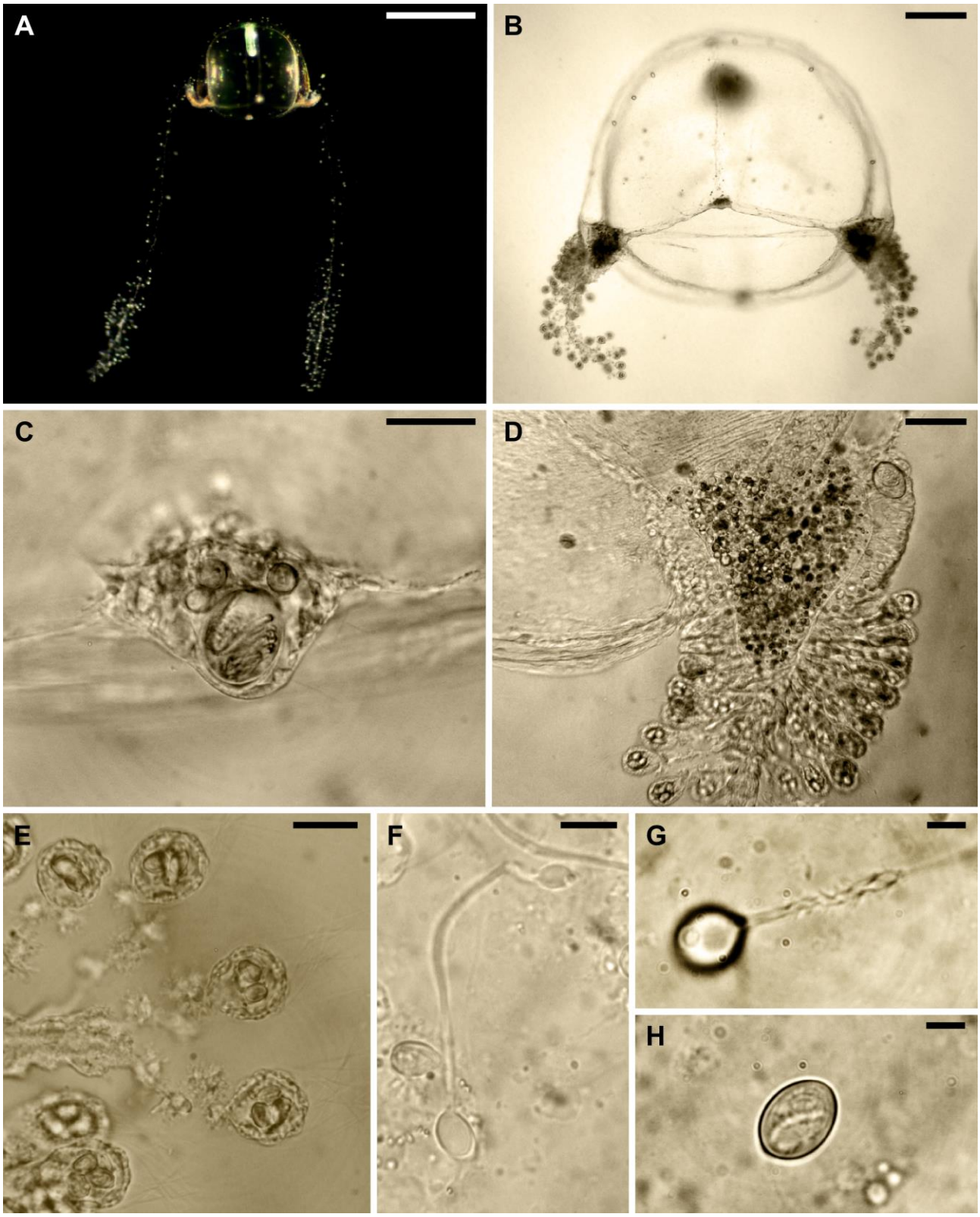

Figure 3.3. Medusa stage of *Pseudozanclea timida*. A) Four days old and B) newly released medusa, with euryteles in the C) exumbrellar nematocyst pouches, as well as in D) tentacular bulbs. E) Cnidophores containing F) bean-shaped euryteles. G, H) Discharged and undischarged euryteles on the exumbrella. Scale bars: A) 0.5 mm, B) 0.1 mm, C-E) 25 μm, F-H) 5 μm.

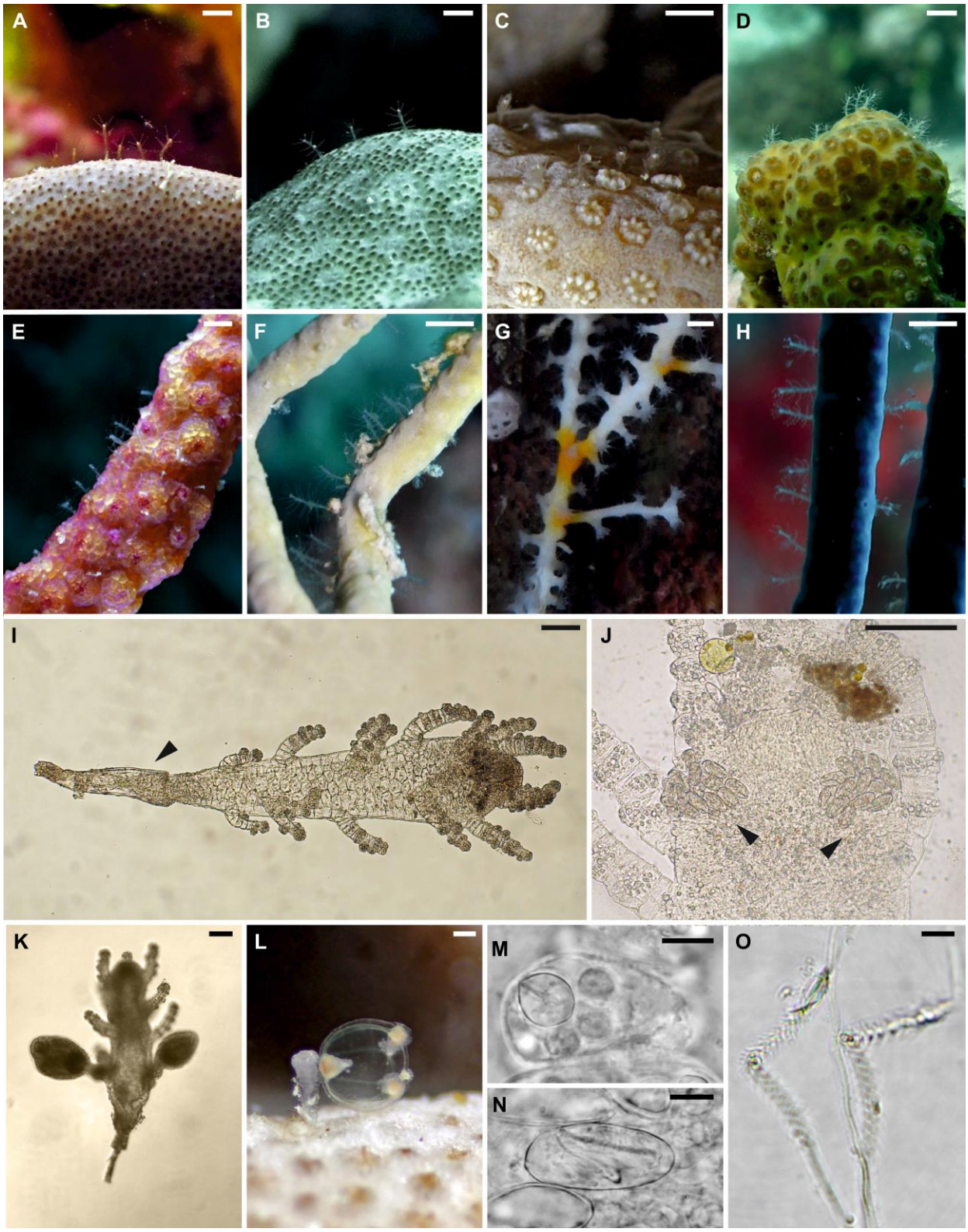

Figure 3.4. Polyp stage of *Pteroclava krempfi*. Colonies associated with A) *Lobophytum*, B) *Sarcophyton*, C) *Sinularia* from Maldives, D) *Rhytisma* from the Red Sea, E) *Paraplexaura* from Maldives, F) *Astrogorgia*, G) *Melithaea* from the Red Sea, and H) *Antillogorgia* from Sint Eustatius. I) Detached polyp showing moniliform tentacles, a perisarc-covered pedicel (arrowhead), and J) clusters of euryteles (arrowheads) under the hypostome. K) Gono-gastrozooid with three medusa buds and L) degenerated polyp with a mature medusa bud (reproductive exhaustion). M) Large and small stenoteles in a capitulum. N) Undischarged eurytele capsule and O) detail of the shaft of a discharged eurytele. Scale bars: A-H) 1.5 mm, I-L) 0.1 mm, M-O) 10 μm.

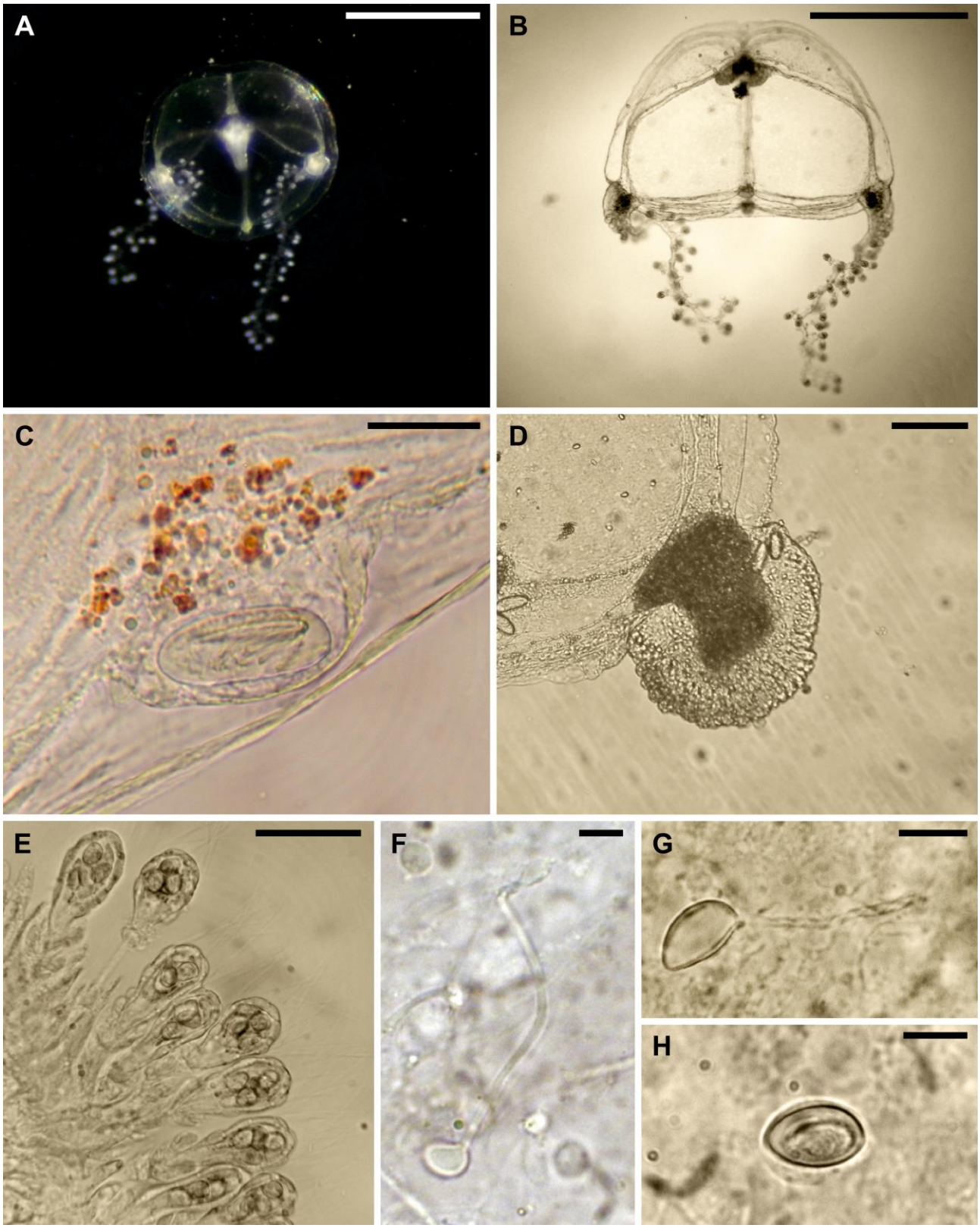

Figure 3.5. Medusa stage of *Pteroclava krempfi*. A) Two days old medusa detached from a polyp associated with *Sinularia* and B) newly liberated medusa detached from a polyp growing on *Paraplexaura*. C) Exumbrellar nematocyst pouches and B) tentacular bulb containing large euryteles. E) Cnidophores containing F) bean-shaped euryteles. G, H) Discharged and undischarged euryteles on the exumbrella. Scale bars: A, B) 0.5 mm, C) 25 μm, D, E) 50 μm, F-H) 5 μm.

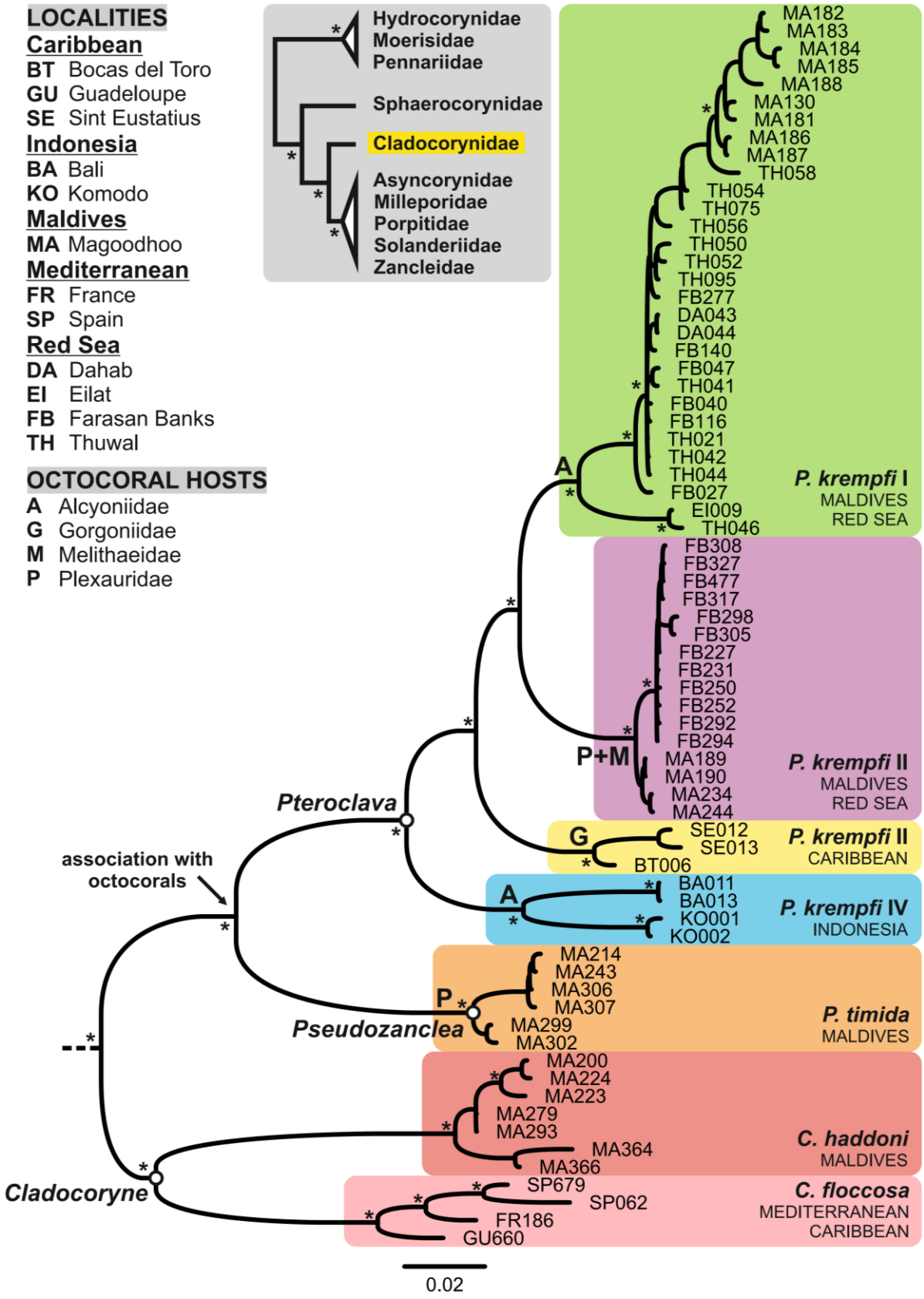

Figure 3.6. Bayesian phylogenetic hypothesis for the Cladocorynidae based on the concatenated mitochondrial and nuclear genes. Fully supported nodes (BPP > 0.95, BS > 95) are indicated with asterisks. Each of the main lineages is highlighted with a different colour. Localities and host families are indicated for each specimen as coded in the legend. Position of the Cladocorynidae within the superfamily Zancleida is shown in the grey box.

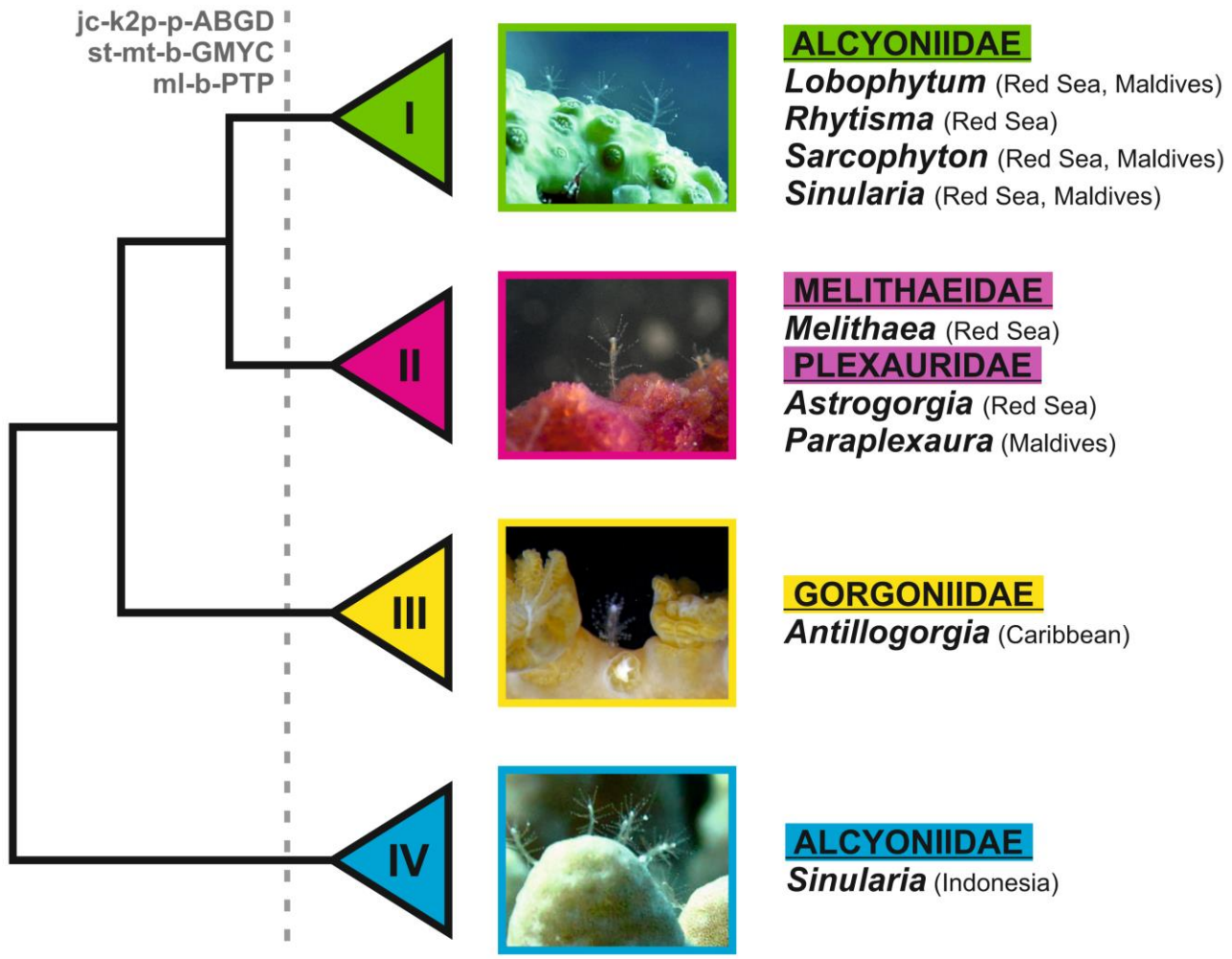

Figure 3.7. Species delimitation hypothesis for the *Pteroclava krempfi* species complex. The four detected cryptic species are coloured as the phylogenetic clades in Figure 3.6. For each species, the genus and family of the hosts, and the geographic distribution are shown on the right.

CHAPTER 4

Phylogeny, diversity and evolution of the family Sphaerocorynidae (Hydrozoa, Capitata)

This work is partially published in:

1. Maggioni D, Galli P, Berumen ML, Arrigoni R, Seveso D, Montano S. (2017). *Astrocoryne cabela* gen. nov., sp. nov. (Hydrozoa: Sphaerocorynidae), a new sponge-associated hydrozoan. *Invertebrate Systematics* 31, 734-746. doi: 10.1071/IS16091.

and in preparation for the submission as:

2. Maggioni D, Galli P, Seveso D, Berumen ML, Arrigoni R, Hoeksema BW, Montano S. Phylogeny, diversity and evolution of the family Sphaerocorynidae (Hydrozoa, Capitata).

4.1. ABSTRACT

The family Sphaerocorynidae includes five valid species, currently subdivided in two valid genera, and with often confusing taxonomic histories. Here, a molecular phylogeny of the family is proposed for the first time, with an assessment of some of the previously known species and the discovery of two new genera and at least one new species. Each of the sampled taxon is described or re-described and new morphological characters to distinguish the different sphaerocorynid species are proposed. Most species do not exhibit evident intra-specific morphological variation, with the exception of the new species and genus *Astrocoryne cabela*, having this organisms differences in populations from the Maldives and the Red Sea that could be related to geographical isolation and different ecological pressures. However, all species show low to moderate levels of genetic structuring, and only populations of the new genus *Sphaerocorynoides* show a clear geographic diversification. The main difference among the polyp stage of all species and genera is the arrangement and type of tentacles. Tentacles can be organised in single or multiple whorls, longitudinally grouped, and also partially fused. Here, a novel type of tentacle is described from *Astrocoryne cabela*, and the possible evolution of different tentacular structures is discussed. Overall, the family Sphaerocorynidae shows a previously overlooked diversity that is likely to be further increased with the inclusion in the analyses of additional sampling localities and other species. Moreover, this work highlights that comprehensive and multidisciplinary studies of the family are needed to clarify the evolution of this group of hydrozoans as well as their relationship with their sponge hosts.

4.2. INTRODUCTION

Hydrozoan polyps are involved in interspecific associations with several other organisms, including cnidarians, sponges, bryozoans, arthropods, annelids, molluscs, echinoderms, chordates, seaweeds, and seagrasses (Gili and Hughes 1995, Boero and Bouillon 2005, Puce et al. 2008). In some cases, hydroids are obligatorily associated with their hosts, and these intimate associations are assumed to be achieved by the selectivity of hydrozoan larvae (e.g. Conover and Sieburth (1964), Piraino et al. (1994), Orlov (1997)). In particular, the drivers of planula settlement of some hydroid species on specific hosts are known to be species-specific bacterial cues, such as for *Hydractinia echinata* (Fleming 1828), for which the larval settlement and metamorphosis on gastropod shells inhabited by hermit crabs are regulated by *Pseudoalteromonas espejiana* (see Müller and Leitz (2002) for a review). Sponges are one of the most common hosts for hydrozoans (Puce et al. 2008), and associations may range from occasional to highly specific (Puce et al. 2005). To date, 26 hydrozoan species are known to live embedded by tissues of 28 species of sponges (Puce et al. 2005, Schuchert and Reiswig 2006, Brinckmann-Voss and Lindner 2008, Puce et al. 2008), whereas more than 100 species have been reported as occasional epibionts (Shimabukuro 2007). The specifically associated species show variable levels of integration with their sponge hosts: i) hydranths grow into the canal system of the sponge; ii) they protrude from the surface, but can retract into the host body; iii) they protrude from the surface and cannot retract into the sponge (Puce et al. 2005). The latter case is represented, for instance, by the family Sphaerocorynidae Prévot 1959, which has hydranths arising from the sponge surface, with the hydrorhiza embedded by host tissues. This family is composed of two genera, *Sphaerocoryne* Pictet 1893 and *Heterocoryne* Wedler & Larson 1986. Although *Sphaerocoryne* is rarely found on other substrates, such as living corals (Calder et al. 2003), coral rubble (Calder 2010), bivalves (Calder 1971), serpulid tubes (Galea 2008), and other hydrozoans (Mergner and Wedler 1977, Shimabukuro et al. 2006), both genera are considered obligate sponge-associated taxa, especially *Heterocoryne*, which is exclusively found on sponges (Wedler and Larson 1986, Galea and Ferry 2013).

The family Sphaerocorynidae was erected by Prévot (1959), and formerly included only *Sphaerocoryne*, which had been assigned to the Corynidae Johnston 1836 by previous authors. *Heterocoryne* was later discovered by Wedler and Larson (1986), and was then assigned to the same family by Petersen (1990). Molecular analyses revealed that Sphaerocorynidae belongs to the clade Capitata *sensu stricto*, and it seems to be one of the basal taxa of the superfamily Zancleida (Nawrocki et al. 2010, Maggioni et al. 2016). However, the analysis of the concatenated *16S*, *18S* and *28S rRNA* included only two *Sphaerocoryne* species and did not provide strong statistical support for their placement within Zancleida, due to either the undersampling or the fact that no *18S rRNA* sequences were obtained for those species.

The taxonomic histories of most species of the family Sphaerocorynidae are confusing and the validity of some taxa still needs to be assessed. To date, four valid species belong to *Sphaerocoryne*, even though for two species, i.e. *Sphaerocoryne coccometra* (Bigelow 1909) and *Sphaerocoryne peterseni* Bouillon 1984, only the medusa stage is known (Bigelow 1909, Bouillon 1984). The remaining two species, *Sphaerocoryne agassizii*

(McCrady 1859) and *Sphaerocoryne bedoti* Pictet 1893, show little morphological variation, and the diagnostic characters are mainly limited to the position of medusa buds in polyps, and the arrangement of nematocysts, the number of tentacles, and the presence of ocelli in medusae at release (Calder 2010). Moreover, these two species were previously assigned to two genera (i.e. *Corynetes* Haeckel 1879 and *Sphaerocoryne*, respectively) and Calder (2010) and Schuchert (2010) suggested to retain the two genera as distinct, at least until a molecular phylogeny clarifies their relationship. However, no conclusions on this issue have been drawn, due to the lack of precise knowledge of life cycles and morphology. *Heterocoryne* contains a single well-established species, *Heterocoryne caribbensis* Wedler & Larson 1986, whose morphological differences with *Sphaerocoryne* are represented by the arrangement of tentacles and the reproductive structures (Wedler and Larson 1986, Petersen 1990). Indeed, *Sphaerocoryne* is characterised by the presence of three to five whorls of capitate tentacles below the hypostome of the polyp, whereas *Heterocoryne* has a distal whorl of simple capitate tentacles and a proximal whorl of trifid capitate tentacles. Regarding the gonosome, *Sphaerocoryne* species reproduce via free-living medusae, whereas *Heterocoryne* bears gonophores reduced to single eumedusoids (Petersen 1990). In this study, the phylogenetic relationships among different taxa of the Sphaerocorynidae are explored using three mitochondrial and three nuclear gene markers, the morphology of previously described and new species and genera is described, and the tentacle evolution is discussed.

4.3. MATERIAL AND METHODS

Colonies of Sphaerocorynidae were collected during several field surveys conducted at Magoodhoo Island, Faafu Atoll, Maldives (MA) from April 2015 to April 2017; St. Eustatius, Dutch Caribbean (SE) in June 2015; Bocas del Toro, Panama (BT) in July 2015; Thuwal (TH) and Farasan Banks (FB), Saudi Arabian Red Sea in December 2015 and May 2017. Fragments of sponges associated with hydroids were sampled using plastic bags by SCUBA diving (maximum depth in all dives: ~30 m) and placed in water tanks just after the dives. Hydroid colonies were reared in laboratory in order to allow the release of medusae, which, when possible, were cultivated for up to one week. Both polyps and newly released medusae were maintained in small oxygenated bowls at room temperature, and water was replaced daily. Samples were fixed in 10% formalin and in 99% ethanol for morphological and molecular characterisations, respectively. A Zeiss Axioskope 40 microscope was used to study the morphology of medusae and polyps, the latter after being carefully detached from their host under a Leica EZ4 D stereo microscope, using syringe needles and micropipettes. All pictures were taken with a Nikon AW100 camera, and measurements were obtained with an ocular micrometer.

DNA from ethanol-preserved samples was extracted following a protocol modified from Zietara et al. (2000). Additional DNA extracts of Sphaerocorynidae from Guadeloupe and Portugal were obtained from the Natural History Museum of Geneva. Six molecular markers were amplified: a portion of the nuclear *28S rRNA* (~1700 bp), *18S rRNA* (~1700 bp), and *ITS* (~1700 bp) and a portion of the mitochondrial *16S rRNA* (~600 bp), *COXI* (~1700 bp), and *COXIII* (~1700 bp). The PCRs were set up using the same protocols and primers described in Maggioni et al. (2016), Medlin et al. (1988), Fontana et al. (2012), Cunningham and Buss (1993), Folmer et

al. (1994), Peña-Cantero and Sentandreu (2017), respectively. All PCR products were purified and directly sequenced in forward and reverse directions using an ABI 3730xl DNA Analyzer (Applied Biosystem, Foster City, CA, USA). The sequences obtained were aligned with other representatives of the Zancleida using MAFFT 7.110 (Katoh and Standley 2013), with the E-INS-i option. The obtained alignments were run through Gblocks (Castresana 2000, Talavera and Castresana 2007) to remove ambiguously aligned regions, using the default 'less stringent' settings. The sequences were combined into a concatenated dataset, and PartitionFinder 1.1.1 (Lanfear et al. 2012) was used to determine the partition scheme and the molecular models. Bayesian inference (BI) and maximum likelihood (ML) were used to infer phylogenetic relationships of both multi- and single-locus datasets. BI analyses were performed using MrBayes 3.2 (Ronquist et al. 2012). Four parallel Markov Chain Monte Carlo runs (MCMC) were run for 10^7 generations. Trees were sampled every 100^{th} generation, and burn-in was set to 25%. ML trees were built with Garli 2.01 (Zwickl 2006) and read into the SumTrees 4.0.0 program in the DendroPy 4.0.0 package (Sukumaran and Holder 2010) to calculate non-parametric bootstrap support values from 1000 replicates, each based on five heuristic search replicates, and to map them on the best ML tree. Bayesian posterior probabilities (BPP) and bootstrap values (BS) were indicated at each node. The genetic distances (uncorrected p-distance, 1000 bootstrap replicates) within and among species were also estimated using MEGA 6 (Tamura et al. 2013).

For selected species (i.e. species with a distribution not limited to a single region) genetic differentiation among populations was tested using analysis of molecular variance (AMOVA) of *16S rRNA* sequences (most complete dataset) in Arlequin 3.5.1.2 (Excoffier and Lischer 2010).

4.4. RESULTS

Throughout the field surveys, 72 colonies of sponge-associated hydrozoans belonging to the family Sphaerocorynidae were collected from the investigated localities. A total of five species were identified on morphological basis and subsequently confirmed through molecular analyses: *Sphaerocoryne* cf. *agassizii*, *Sphaerocoryne bedoti*, *Heterocoryne caribbensis*, *Sphaerocorynoides* sp. (gen. nov.) and *Astrocoryne cabela* (gen. et sp. nov.). Molecular phylogenetics allowed to build a well-supported phylogenetic hypothesis that showed the presence of two previously undescribed genera and at least one new species. The molecular diversity and phylogenetic relationships are described in the following paragraph 'Molecular Analyses', whereas the morphology of all the species is described in the 'Morphological Analyses' paragraph.

4.4.1. Molecular analyses

The total alignment of the concatenated *16S-COX1-COXIII-18S-28S-ITS* dataset was 5678 bp long after Gblock treatment, and included 389 sequences generated from the 72 specimens collected, plus 65 sequences from other representatives of all the main families belonging to the superfamily Zancleida and *Stauridiosarsia nipponica* (Uchida 1927) and *Coryne pintneri* Schneider 1897 (outgroups). The general topology of the phylogenetic trees based on BI and ML analyses were almost identical, and therefore only the Bayesian

topology is shown (Figure 4.1). The overall statistical support of the tree is higher in the concatenated analysis, with almost all nodes supported by BPP > 0.95 and BS > 95, and this analysis is therefore considered as the most robust phylogenetic hypothesis. The results are concordant with previous works (Nawrocki et al. 2010, Maggioni et al. 2016), recovering the superfamily Zancleida as a monophyletic lineage, with the clade composed of Pennariidae McCrady 1859, Hydrocorynidae Rees 1957, and Moerisiidae Poche 1914 being the sister group of the remaining families. All the specimens belonging to Sphaerocorynidae form a fully supported monophyletic group. This family diverged early from the other taxa of the Zancleida, just after the Pennariidae-Hydrocorynidae-Moerisiidae group. Moreover, the addition of new taxa and molecular markers to the dataset improved the statistical support of this node.

Regarding the relationships within the Sphaerocorynidae, five fully supported clades, corresponding to as many species, are recovered (Figure 4.1). The two *Sphaerocoryne* species included in the analyses (i.e. *Sphaerocoryne* cf. *agassizii* and *Sphaerocoryne bedoti*) are here sister species, but the node is not fully supported (BPP = 0.7 and BS = 79). The *S.* cf. *agassizii* clade is composed of two subclades, one including specimens from the Indian Ocean (Maldives) and the Caribbean Sea (Sint Eustatius), and the second one including only specimens from the Caribbean (Panama). Specimens of *S. bedoti* were collected from Indian Ocean (Maldives), Red Sea (Saudi Arabia), Caribbean Sea (Panama and Sint Eustatius) and Eastern Atlantic Ocean (Portugal) and showed no further clear genetic subdivision. *Heterocoryne caribbensis* is the sister species of the two *Sphaerocoryne* species and specimens from Sint Eustatius and Guadeloupe cluster together in a clade with maximal support. A fourth clade is represented by specimens collected from the Red Sea (Saudi Arabia) and Indian Ocean (Maldives), belonging to a new genus here named *Sphaerocorynoides* (see 'Morphological Analyses' paragraph) and specimens from the two locality cluster in two phylogenetically distinct cades. Finally, an additional new genus and species is represented by specimens collected in the Indian Ocean (Maldives) and Red Sea (Saudi Arabia). This taxon, here named *Astrocoryne cabela* (see 'Morphological Analyses' paragraph), represents the earliest diverging group within Sphaerocorynidae and specimens from the two localities appear to be moderately divergent.

Genetic distances among species are high for all mitochondrial markers and for the nuclear *ITS*, whereas, the *18S* and *28S rRNA* show lower values (Table 4.1). Intra-specific genetic divergence is relatively high for *Sphaerocoryne* species, especially for mitochondrial markers, suggesting a further genetic diversification at population level, whereas it is generally low for the other species (Table 4.1). However, AMOVA found significant geographic diversification only between population of *Sphaerocorynoides* sp. from Maldives and Red Sea (*Fst* = 0.89, p = 0.0000), whereas for *S.* cf. *agassizii* and *S. bedoti* populations no relevant geography-related genetic structure seems to occur (*Fst* = 48.56 and 37.92, respectively - p = 0.0000).

4.4.2. Morphological analyses

Family Sphaerocorynidae Prévot 1959

Amended diagnosis: Colony stolonal, hydrorhiza creeping, hydrocaulus long, unbranched, or sparingly branched, with terminal hydranths. Perisarc thin, reaching hydranth base or stopping at the insertion with the hydrorhiza. Hydranth pyriform, with bulbous base and proboscis-like hypostome, no oral tentacles, but single, trifid or dicapitate tentacles in one to five whorls around broadest part of body. Gonophores arising above or among tentacles as free medusae or sessile eumedusoids.

Reproduction via eumedusoid or free medusa with thick bell-shaped or conical umbrella. Apical projection, when present, conical or dome-shaped, with thick mesoglea and broad apical chamber. Manubrium flask-shaped, quadrate, or cruciform, narrowing towards the mouth, the latter being simple, round or cruciform. Two or four tentacles with adaxial, evenly distributed, spirally arranged or absent nematocyst clusters, with a terminal, ellipsoid or spherical capitation. Marginal bulbs large, clasping exumbrella, each with adaxial expansion. Ocelli abaxial, not always present in newly liberated medusae. Gonads adradial, confluent in perradii in mature specimens.

Genus ***Sphaerocoryne*** Pictet 1893

Diagnosis: Colony stolonal, hydrocaulus long, simple or slightly branched, hydranth vasiform, with numerous simple solid capitate tentacles in 3-5 whorls around broadest part, gonophores on short branching blastostyles above or among tentacles. Reproduction via medusa (see family characters).

Sphaerocoryne* cf. *agassizii (McCrady 1859)

Figure 4.2

Polyp: Colonies monomorphic, living in association with different sponges. Hydrorhiza tubular, branched, covered by perisarc and growing in sponge tissues. Pedicels long (up to 5 mm), unbranched, covered by a smooth, thin perisarc. Hydranth pyriform, up to 2.5 mm long, with variable diameter (150-420 μm) (Figure 4.2A-C). Hypostome proboscis-like, contractile, with a band of nematocysts below the mouth. Up to 45 tentacles arranged in 3-5 close whorls in the broadest part of the polyp, and grouped longitudinally (Figure 4.2D. Each tentacle with a terminal, nematocyst-rich capitation (diameter: 100-140 μm in the distal whorls; 85-95 μm in the proximal whorl) (Figure 4.2E-H). Tentacles up to 600 μm long in the distal whorls, shorter in the proximal whorl. Up to seven medusa buds at the same stage of maturation develop above distal tentacles (Figure 4.2C), singly on blastostyles. Living hydranths with white mouths, an irregular yellow-orange band below the hypostome, at the level of the gastric cavity, and white, clearly visible, mesenteric filaments (Figure 4.2B, C). Desmonemes, small and large stenoteles occurring simultaneously and concentrated in the capitula,

and, more rarely, scattered in the hydrorhiza and in the hydranth; small stenoteles in a band around the hypostome.

Medusa: Not observed.

Cnidome: i) Desmonemes (undischarged: 10 x 4-5 μm; discharged capsule: 8 x 5 μm; shaft: 4 x 5 μm). ii) Large stenoteles (undischarged: 22-24 x 12-15 μm; discharged capsule: 19-21 x 12-13 μm) iii) Small stenoteles (undischarged: 11-14 x 8-10 μm; discharged capsule: 10 x 7-8 μm).

Remarks: The specimens here described are provisionally ascribed to the species *Sphaerocoryne agassizii* on the basis of the polyp morphology, since the mature medusa was not observed. Differently from *Sphaerocoryne bedoti*, the polyp shows tentacles more or less longitudinally grouped, medusa buds organised singly, and a typical colouration, consisting in white mouth and mesenteric filaments and an irregular yellowish band below the hypostome. *Sphaerocoryne* cf. *agassizii* is here reported for the first time from the Indian Ocean and Caribbean Sea, thus widening the distribution of the species, which was previously reported only from the Atlantic coasts of North America (McCrady 1859, Hargitt 1904, Hargitt 1908, Calder 1971, Petersen 1990) and from Panama and Ecuador in the Pacific Ocean (Fraser 1938), even if the latter report needs to be confirmed. Some doubts remain about the identification of these specimens due to the medusa-based previous descriptions and to the fact that the polyp stage of this species was often confused with the polyp of *S. bedoti*.

Sphaerocoryne bedoti Pictet 1893

Figure 4.3

Polyp: Colonies monomorphic, living in association with different sponges. Hydrorhiza tubular, branched, covered by perisarc and growing within sponge tissues. Pedicels long (up to 4 mm), unbranched, covered by a slightly wrinkled, thin perisarc. Hydranth pyriform, up to 3 mm long, with variable diameter (130-510 μm) (Figure 4.3A-C). Hypostome proboscis-like, contractile, with a band of nematocysts below the mouth. Up to 37 tentacles with no evident arrangement and closely scattered in the broadest part of the hydranth (Figure 4.3D). Each tentacle with a terminal, nematocyst-rich capitation (diameter: 110-140 μm in the distal whorls; 70-85 μm in the proximal whorl), with a light-reflecting inclusion (Figure 4.3F-I). Tentacles up to 550 μm long in the distal whorls and shorter in the proximal whorl. Up to five clusters of 5-20 medusa buds at the same stage of maturation develop above distal tentacles, in the correspondence of a red band (Figure 4.3C). Living hydranths with white hypostome, a regular bright red band below the hypostome and a gastric cavity transparent or yellowish in the broadest part and withish below the broadest part of polyps (Figure 4.3A, B). Desmonemes, small and large stenoteles occurring simultaneously and concentrated in the capitatula, and, more rarely, scattered in the hydrorhiza and in the hydranth; heteronemes in the hydrocaulus and hydrorhiza; small stenoteles in a band around the hypostome.

Medusa: Newly liberated medusae small, 105-170 μm wide and 110-200 μm high, with nematocysts scattered on the exumbrella (Figure 4.3E). Manubrium short, 40-50 μm long and 80-110 wide at the base, with a circular mouth, with no incipient gonads. No radial canals visible, four small triangular bulbs. When released, medusae

with no tentacles, six days after release, medusae of the same size, with four short tentacles, 40-50 μm long. Living medusae transparent with reddish bulbs. Desmonemes and small stenoteles scattered on the exumbrella. *Cnidome*: i) Desmonemes (undischarged: 10-12 x 5-6 μm; discharged capsule: 10-11 x 5-6 μm: shaft: 7 x 7-8 μm). ii) Heteronemes (undischarged: 20-22 x 8-10 μm). iii) Large stenoteles (undischarged: 25-28 x 18-20 μm; discharged capsule: 20-21 x 14-16 μm). iv) Small stenoteles (undischarged: 10-12 x 8-10 μm; discharged capsule: 10 x 6 μm).

Remarks: The analysed specimens are here assigned to *S. bedoti* due to the polyp morphology, specifically due to the colouration of the polyp and the organisation of medusa buds. Moreover newly released medusae are similar to the one described by Yamada and Konno (1973). The morphological features that distinguish *S. bedoti* from *S. agassizii* are the tentacles not longitudinally organised, the light-reflecting inclusions in capitula, the medusa buds grouped in clusters, and the characteristic colouration of the polyp, consisting in a bright red band below completely white hypostomes. Moreover, an additional type of nematocyst (i.e. heteroneme) previously overlooked is described from the pedicel and hydrorhiza. Previous descriptions and identifications of *S. bedoti* are often confusing and only a part of the previous reports of this species can be confirmed on the basis of the morphology and coloration of the polyp stage. According to this fact and to our findings, the distribution range of the species is here confirmed for the following localities: Bermuda, Guadeloupe, Panama, Puerto Rico, Sint Eustatius, Portugal (Atlantic Ocean); Indonesia, Colombia, Japan (Pacific Ocean); South Africa, India, Maldives (Indian Ocean); Saudi Arabia (Red Sea) (Pictet 1893, Warren 1908, Fraser 1938, Millard 1975, Wedler and Larson 1986, Calder 1988, Hirohito 1988, Galea 2008, Nagale and Apte 2014).

Genus ***Heterocoryne*** Wedler & Larson 1986

Diagnosis: Colony stolonal, hydrocaulus unbranched, hydranth vasiform, with one whorl of simple long capitate tentacles and one whorl of long trifid capitate tentacles closely-set around broad basal part, gonophores single, on short pedicel, among upper whorl of simple capitate tentacles, reduced to eumedusoids.

Heterocoryne caribbensis Wedler & Larson 1986

Figure 4.4

Polyp: Colonies monomorphic, living in association with the sponge *Mycale* sp. Hydrorhiza tubular, branched, covered by perisarc and growing within sponge tissues. Pedicels unbranched, covered by a thin perisarc and by sponge tissues, forming a cone-shaped cover around the base of the polyp. Hydranth pyriform, up to 3.5 mm long, with variable diameter (160-630 μm) (Figure 4.4A, B). Hypostome proboscis-like. One whorl of up to eight capitate tentacles below the hypostome and one whorl of up to 12 aboral capitate tentacles, each one provided with two partially-fused capitate tentacles on the proximal side (Figure 4.4C). Each tentacle with a terminal, nematocyst-rich capitation (diameter: 95-130 μm), with a light-reflecting inclusion (Figure 4.4C-F). Tentacles up to 1 mm long. One gonophore (eumedusoid) per polyp, among oral tentacles. Living hydranths

with white hypostome, and a reddish gastric cavity (Figure 4.4A, B). Desmonemes, small and large stenoteles occurring in the capitations, and, more rarely, scattered in the hydrorhiza and, more rarely, in the hydranth.
Cnidome: i) Desmonemes (undischarged: 8-9 x 4-5 μm; discharged capsule: 7 x 4-5 μm; shaft: 4 x 5 μm). ii) Large stenoteles (undischarged: 18-19 x 12-13 μm; discharged capsule: 17 x 11-10 μm). iii) Small stenoteles (undischarged: 10-12 x 6-7 μm; discharged capsule: 8-9 x 5 μm).
Remarks: The colonies investigated in this study are morphologically identical to the specimens described by Wedler and Larson (1986) and Galea (2013). The updated distribution of this species in the Caribbean Sea is limited to the North-Eastern side and includes Puerto Rico, Guadeloupe, and Sint Eustatius.

Genus ***Sphaerocorynoides*** gen. nov.

Diagnosis: Colony stolonal, hydrocaulus short, hydranth vasiform, with solid capitate tentacles in two close whorls around broadest part, gonophores on short blastostyles above tentacles. Reproduction via medusa.
Etymology: Sphaerocorynoides derives from the combination of the generic name *Sphaerocoryne* and the Greek suffix *-oides* (like), due to the resemblance of the genus to *Sphaerocoryne.* It is a masculine noun.

***Sphaerocorynoides* sp.**

Figure 4.5

Polyp: Colonies monomorphic, living in symbiosis with different sponges, and occasionally sharing the host with *Astrocoryne cabela*. Hydrorhiza tubular, branched, covered by thin perisarc, crawling within sponge tissues. Pedicels short (up to 180 μm), unbranched, covered by a thin perisarc, often overgrowth by sponge tissues. Hydranth pyriform, up to 0.7 mm long, with variable diameter (50-230 μm) (Figure 4.5A-C). Hypostome proboscis-like, contractile. Up to 22 tentacles arranged irregularly in two very close whorls in the broadest part of the polyp (Figure 4.5D). Each tentacle with a terminal, nematocyst-rich capitation (diameter: 70-100 μm) (Figure 4.5F-J). Tentacles 100-300 μm long. Up to two medusa buds at different stages of maturation develop above tentacles, singly on blastostyles (Figure 4.5C). Living hydranths transparent, with white mouths (Figure 4.5A-C). Desmonemes, small and large stenoteles occurring in the capitations, as well as scattered in the hydrorhiza; macrobasic mastigophores occurring in the pedicel and hydrorhiza.
Medusa: Newly liberated medusa with a bell-shaped umbrella, 370-410 μm wide and 405-420 μm high, with several nematocysts scattered on the exumbrella (Figure 4.5E). Manubrium cylindrical, about 300 μm long, 2/3 to 3/4 of the bell height, distally provided with a circular mouth. Four radial canals end in four bulbs with a diameter of 70-80 μm, containing nematocysts. When released, medusae with no tentacles and no ocelli. Living medusae transparent with reddish manubria (Figure 4.5C). Nematocysts scattered on the exumbrella (microbasic mastigophores and small stenoteles), and in bulbs (large stenoteles).
Cnidome: i) Desmonemes (undischarged: 9-10 x 4-5 μm; discharged capsule: 6 x 4 μm; shaft: 5 x 3 μm). ii) Macrobasic mastigophores (undischarged: 10-11 x 5-6 μm; discharged capsule: 8-9 x 5 μm; shaft: 45-55 μm).

iii) Microbasic mastigophores (undischarged: 8 x 4-5 μm; discharged capsule: 7 x 4 μm; shaft: 5-8 μm) iv) Large stenoteles (undischarged: 18-20 x 12-14 μm; discharged shaft: 16-17 x 11 μm). v) Small stenoteles (undischarged: 10-13 x 7-10 μm; discharged capsule: 7-11 x 5-8 μm).

Remarks: The analysed samples are placed in the newly erected genus *Sphaerocorynoides* mainly due to phylogenetic analyses. However, this taxonomic decision is also supported by morphological differences with other sphaerocorynid species and genera: the two close whorls of tentacles, the short pedicel, the transparent hydranth, and the presence of macrobasic mastigophores in the pedicel and hydrorhiza of the polyps. The fact that the adult medusa of this species was not observed did not allow to take a taxonomic decision for the identification at species level. Indeed, the medusa of *Sphaerocorynoides* sp. could correspond to one of the two *Sphaerocoryne* species previously described on the basis of the medusa stage and for which the polyp stages remain unknown (i.e. *Sphaerocoryne peterseni* and *Sphaerocoryne coccometra*). The distribution range for this species is for now limited to the Maldives and the Saudi Arabian Red Sea but further field surveys are likely to extend this range.

Genus ***Astrocoryne*** gen. nov.

Diagnosis: Colony stolonal, hydrocaulus short to moderately long, hydranth slightly vasiform, with dicapitate tentacles in one or two alternating whorls around broadest part, gonophores on short blastostyles among tentacles. Reproduction via medusa.

Etymology: Astrocoryne derives from the combination of the Greek *Aster*, meaning star and referring to the star-shaped aspect of the hydranth, and *Coryne*, following the structure of names of the other genera ascribed to Sphaerocorynidae. It is a feminine noun.

Astrocoryne cabela gen. et sp. nov.

Figure 4.6

Polyp: Colonies monomorphic, living in association with different sponges. Hydrorhiza tubular, branched, covered by moderately thick and slightly wrinkled perisarc, embedded by sponge tissues and giving rise to several hydranths. Pedicels short (90-190 μm) to moderately long (450-580 μm), unbranched, covered by a smooth, thin, cup-shaped or elongated perisarc. Hydranth slightly pyriform, up to 0.8 mm long, with variable diameter (90-240 μm) (Figure 4.6A-C). Hypostome proboscis-like, contractile. Up to 10 tentacles (generally 8-10 occur) arranged in either one (specimens from Maldives) or two close whorls (specimens from the Red Sea) in the broadest part of the polyp (Figure 4.6C). Each tentacle with a terminal, nematocyst-rich capitation (diameter: 50-85 μm in the distal whorl; 25-30 μm in the proximal whorl) and another, sub-terminal, spherical cluster of nematocysts (diameter: 70-110 μm in the distal whorl; 35-45 in the proximal whorl) (Figure 4.6D, F-H). Tentacles 350-530 μm long in the distal whorl, shorter in the proximal whorl (200-320 μm) when present. Nematocyst clusters about 100 μm distant from one other, moved closer by the contraction of the distal part of

tentacles. Up to 11 medusa buds at different stages of maturation develop among tentacles, singly or in couple on blastostyles (Figure 4.6C, E). Living hydranths transparent, with white mouths and whitish or light orange gastric cavities (Figure 4.6A, B). Desmonemes, microbasic euryteles, small, large and medium stenoteles occurring simultaneously and concentrated in the terminal and proximal capitations, as well as scattered in the hydrorhiza, more rarely in the hydranth.

Medusa: Newly liberated medusa hemispherical, 460-500 μm wide and 480-500 μm high, with nematocysts scattered on the exumbrella (Figure 4.6I, J). Manubrium cylindrical, 150-200 μm long and 80-110 wide at the base, spanning from 1/3 to 1/2 of the bell height, distally provided with a circular mouth (diameter 50-75 μm). Four radial canals end in four bulbs with a diameter of 60-70 μm, linked by a circular canal. Both bulbs and circular canal contain nematocysts. When released, medusae with no tentacles, but all bulbs show swellings filled with nematocysts (Figure 4.6I). Two days after release, medusae with two opposite tentacles (Figure 4.6J, K). Tentacles up to 300 μm long, armed with terminal spherical nematocyst-rich capitations with diameter of 70-85 μm (Figure 4.6K). Ocelli not present at release. Nematocysts scattered on the exumbrella (microbasic mastigophores and rarely microbasic euryteles similar to those found in polyps), in the circular canal (medium-sized stenoteles), in the bulb swellings and in the terminal capitations of tentacles (medium-sized stenoteles and desmonemes).

Cnidome: i) Desmonemes (undischarged: 7-9 x 4-5 μm; discharged capsule: 6-8 x 4-5 μm; shaft: 5 x 5 μm). ii) Microbasic euryteles (undischarged: 13-15 x 5-6 μm; discharged capsule: 10-12 x 4-5 μm; shaft: 9-11 μm). iii) Large stenoteles (undischarged: 18-21 x 13-17 μm; discharged capsule: 15-18 x 11-15 μm). iv) Medium-sized stenoteles (undischarged: 9-10 x 6-7 μm; discharged capsule: 8-9 x 5 μm). v) Small stenoteles (undischarged: 5-6 x 4-5 μm; discharged capsule: 5 x 4-5 μm). vi) Microbasic mastigophores (undischarged: 6-7 x 5-7 μm; discharged capsule: 5 x 5 μm; shaft: 5 μm).

Remarks: The new species and genus *Astrocoryne cabela* differs from other sphaerocorynids according to the arrangement and type of tentacles in polyps. The species *Bibrachium euplectellae* (Schulze 1880), a poorly-known sponge-associated hydroid, has two opposite capitate tentacles with an adoral cluster of nematocysts just below the terminal capitation (Schulze 1880), the latter feature being similar to *A. cabela*. However, in *B. euplectellae*, the cluster of nematocysts below the capitation is restricted to the adoral side, the polyp has only two tentacles, and there is no information regarding its reproduction. Also the species *Tricyclusa singularis* (Schulze 1876) has tentacles similar to those of *A. cabela*, but the proximal cluster of nematocysts is often incomplete and, in some cases, three intermediate clusters, besides the terminal one, can be found (Vervoort 1949), thus resembling imperfect moniliform tentacles. Regarding the medusa stage, *A. cabela* is similar to the genus *Dicnida* Bouillon 1978, by having a simple mouth, four bulbs, two opposed tentacles with a terminal knob of nematocysts, and an identical cnidome. However, *Dicnida* has a prominent apical projection also in juveniles, the manubrium is quadrate, tentacles are (exceedingly) long and particularly rigid, and in some cases bear halfway secondary ramifications, each with its own terminal capitation, non-tentaculate bulbs are extending on the exumbrella, and finally, both the polyp stage and the size and shape of nematocysts are not known (Bouillon 1978). Another similar medusa is produced by the genus *Eucodonium* Hartlaub 1907,

especially regarding the tentacles (Schuchert 1996, Lin et al. 2016), but this filiferan genus does not have for instance stenoteles, even if the cnidome has been analysed only in the type species *Eucodonium brownei* Hartlaub 1907 (Schuchert 1996) and not in the recently described other species (Lin et al. 2016).

Specimens of *A. cabela* from the Red Sea and Maldives show a variation in the arrangement of tentacles and in the length of the pedicel. Specimens from Maldives have one whorl of tentacles, whereas polyps from the Red Sea have two whorls of tentacles. However, they both share the apomorphy of having a proximal cluster of nematocyst in all tentacles and this new type of tentacle is here named "dicapitate". The perisarc in Maldivian specimens stops at the base of polyps, resulting in a short pedicel, whereas in the Red Sea samples the pedicel is longer and the perisarc almost reaches the proximal whorl of tentacles. Since the colonies found in the Red Sea were not fertile, the medusa was not observed, leaving thus uncertainties about other possible morphological differences in the two populations in this life-cycle stage.

Etymology: The species name *cabela* refers to the name of the house where the parents of DM live, in recognition of their support over the years.

4.5. DISCUSSION

Sponges provide habitats for a large number of species belonging to several taxa (Wulff 2006) and, due the richness of their associated fauna, they have been called 'living hotels' (Pearse 1950). Many of the associated organisms live inside the canal systems, where they can find food and shelter (Tyler and Böhlke 1972, Rützler 1978), whereas others live epibiontically on their outer surface where, for instance, they might benefit from the water renewal generated by the sponge itself (e.g. Uriz et al. (1992)). Only two species of hydrozoans are known to live as sponge endosymbionts and correspond to *Bibrachium euplectellae* and *Brinckmannia hexactinellidophila* Schuchert & Reiswig 2006, both associated with hexactinellid sponges (Schulze 1880, Schuchert and Reiswig 2006). However, most of the sponge-associated hydrozoans can be considered as partial endosymbionts, since their hydrorhizae are surrounded by the host and the hydranths protrude from sponge tissues (Puce et al. 2005). The families Tubulariidae Goldfuss 1818, Corynidae, and Sphaerocorynidae count the highest number of sponge-associated species, but only the latter is known to have all its members specifically associated with sponges (Puce et al. 2005). This idea is here confirmed by the description of the two new sphaerocorynid genera *Astrocoryne* and *Sphaerocorynoides*, which were exclusively found in association with sponges.

The hydroids of *Astrocoryne cabela* and *Sphaerocorynoides* sp. were clearly assignable to the Sphaerocorynidae due to their morphology and, in particular, to the pyriform shape of the hydranth, the proboscis-like hypostome, and the tentacles arranged in one or a few whorls below the hypostome, among or above which the gonophores arise. However, *A. cabela* shows an apomorphy, represented by the peculiar type of tentacles, whereas the most distinguishing traits in *Sphaerocorynoides* sp. are the tentacles in two close whorls and the short pedicel. These differences, along with molecular results, made necessary to create the two new genera. Indeed, phylogenetic analyses confirmed the position of these taxa within the Sphaerocorynidae

and showed a high genetic divergence from *Sphaerocoryne* species and *Heterocoryne caribbensis*, supporting the establishment of the new genera.

According to both morphological and phylogenetic analyses, *Sphaerocoryne* cf. *agassizii* and *Sphaerocoryne bedoti* are clearly separated but closely related. In particular, an effective way to distinguish the polyps of the two species is to look at tentacle organisation and, more easily, to polyp colouration. Both species have typical and conserved patterns of colouration of the mouth, the hypostome, and the body of the hydranth and this trait makes possible a confident identification of the two species also underwater or in highly contracted specimens. Similarly, *Sphaerocorynoides* sp. can be quickly identified thanks to the transparent body of the polyp and the very short pedicel in comparison to the other sphaerocorynid species. Calder (2010) and Schuchert (2010) highlighted the nomenclature problem regarding the genus *Sphaerocoryne*. Indeed, *S. agassizii* was previously included in the genus *Corynetes* (Haeckel 1879), and since this name predates *Sphaerocoryne*, both *Sphaerocoryne* and *Corynetes* were retained valid until a molecular phylogeny would have clarified the relationship between *S. agassizii* and *S. bedoti*. Here, the two species are treated as congeneric and the name *Corynetes* should therefore be used. However, the two authors also stated that a case could be submitted to the International Commission on Zoological Nomenclature (ICZN) asking for a ruling on the merits of conserving the widely used name *Sphaerocoryne* instead of the poorly known *Corynetes*. In this work, the name *Sphaerocoryne* is maintained as valid for both *S. agassizii* and *S. bedoti,* until a decision will be taken by the ICZN.

With a few exceptions, each sphaerocorynid species shows a conserved morphology with highly similar sizes and anatomy in all analysed specimens. The highest morphological variation is found in populations of *A. cabela* from the Red Sea and Maldives. The differences in these populations are represented by the number of whorls of tentacles and the size of pedicels and may be explained by intra-specific morphological variation, a common phenomenon in hydrozoans that can be related to environmental conditions and ontogeny (Cunha et al. 2016). It is likely that unexplored biotic and abiotic ecological factors might contribute, synergistically or not, to the observed morphological variation. However, in other species with populations living in the Red Sea and Maldives (*S. bedoti* and *Sphaerocorynoides* sp.), such morphological variation is not observed and doubts remain about the causes of these differences. Indeed, the Red Sea is known to be a hotspot of biodiversity and endemism (Hughes et al. 2002), and is characterised by a peculiar geological history and several barriers (see DiBattista et al. (2016) for a review) that might be responsible for an ongoing speciation between populations of *A. cabela* from Maldives and Saudi Arabia. Regarding the genetic structure of these species, both *A. cabela* and *Sphaerocorynoides* sp. show a certain divergence between the populations from the two localities, also confirmed by AMOVA in *Sphaerocorynoides* sp., and this may support the hypothesis of an ongoing independent evolution of the lineages in the two localities. Also *S.* cf. *agassizii* is characterised by a moderately high intra-specific genetic diversity, with a value of more than 3 % in the *16S rRNA*, but AMOVA did not reveal a genetic structure related to geography in this species as well as in *S. bedoti*.

The mode of reproduction varies within the Sphaerocorynidae, and differences can be found also in the position and organisation of medusa buds on polyps. Indeed, *H. caribbensis* reproduces via the more derived

eumedusoids, whereas all other species give rise to medusae. The medusa buds are generally organised singly or in few small clusters, with the exception of *S. bedoti*, in which each cluster can include up to 20 buds. In this latter species, released medusae were reared for seven days and showed almost no variation in size and morphology, remaining very small and suggesting a long maturation time. This may suggest the existence of opposed reproductive strategies for *S. bedoti* and the other medusa-producing sphaerocorynids.

The type and organisation of tentacles represents the main difference among the hydranths belonging to different genera of Sphaerocorynidae (Petersen 1990) (Figure 4.7), and both *Astrocoryne* and *Heterocoryne* bear apomorphic tentacles. In *Sphaerocoryne* and *Sphaerocorynoides*, the tentacles are simple capitate and, in *S.* cf. *agassizii* are grouped in longitudinal rows. Tentacles of the proximal whorl in *H. caribbensis* consist of three capitate tentacles partially fused longitudinally in their proximal halves, but still showing their own rows of endodermal cells (Wedler and Larson 1986, Galea 2013), slightly resembling the ramified capitate tentacles of *Cladocoryne*, the latter having nevertheless the endoderm of the axis of tentacle and ramifications fused (Prévot 1959). Finally, *A. cabela* has capitate tentacles with a proximal cluster of nematocysts resembling a halfway capitulum. According to Prévot (1959), the most ancestral state of tentacles is represented by a nematocyst button, followed by the capitate tentacle, and all other type of tentacles evolved from this condition (Figure 4.8). Following this classification, *Sphaerocoryne* and *Sphaerocorynoides* have the most ancestral tentacles among Sphaerocorynidae, having all simple capitate tentacles, whereas *Astrocoryne* and *Heterocoryne* have more derived tentacles. The double capitation in *Astrocoryne* polyps may have derived from a duplication event in the proximal-distal axis of tentacles. Less-regulated duplication events may be responsible for other peculiar tentacles, such as those of *Tricyclusa singularis*, and repeated events may be the explanation for moniliform tentacles. The unique condition of proximal tentacles in *Heterocoryne* polyps may be either the result of partial duplication events along the mid-later axis of capitate tentacles or the partial fusion of already divided tentacles. Therefore, detailed histological analyses, as well as the study of the expression patterns of homeotic genes involved in the development of tentacles, are needed to shed light on the evolution of tentacles in both sphaerocorynid and other capitate hydrozoans.

The association between hydroids and their hosts is usually constant (Puce et al. 2005) and, in some cases, molecular analyses demonstrated that hydrozoan species associated with different hosts actually constitute species complexes, with different molecular groups associated with specific hosts (Maggioni et al. in preparation) . Sphaerocorynid polyps have been mostly reported growing on unidentified sponges, and only in a few cases the identification of the host was provided. In particular, *S. bedoti* is known to be associated with at least nine sponge genera and as many species (Calder 1971, Yamada and Konno 1973, Wedler and Larson 1986, Varela 2012), whereas *H. caribbensis* has been reported so far to live in association with sponges belonging to the genus *Mycale* (Wedler and Larson 1986). Throughout the surveys, each sphaerocorynid species was found associated with a variety of sponges, with the exception of *H. caribbensis*, which was constantly associated with sponges belonging to the genus *Mycale.* In one case, *A. cabela* was found sharing the same sponge host together with a colony of *Sphaerocorynoides* sp. in the Red Sea, suggesting that these species could be generalist symbiont of poriferans. Therefore, according to the literature and our results,

hydrozoans ascribed to Sphaerocorynidae do not seem to have evolved species-specific relationships with their hosts, although further in-depth multi-disciplinary analyses of these associations are needed to address this question. For instance, sponge-associated microorganisms may have a role in the settlement and metamorphosis of sphaerocorynid planulae, similarly to what happens for *Hydractinia echinata* (Müller and Leitz 2002), and microbiome-related studies may help identifying specific shared microbial components among different sponges.

In conclusion, the molecular phylogeny of the Sphaerocorynidae obtained with this work made possible a taxonomic revision of the family, along with the re-description of previously known species and the description of new species and genera. Several evolutionary, ecological, and morphological aspects of these species still need to be assessed, especially regarding the evolution of certain traits and the symbiotic associations with poriferans. Indeed, no information is available about the nature of these association and how they could benefit or damage one or both the symbionts.

4.6. REFERENCES

Bigelow HB (1909) The Medusae. Reports on the scientific results of the expedition to the eastern tropical pacific, in charge of Alexander Agassiz, by the U. S. Fish Commission steamer '"Albatross'" from October, 1904, to March, 1905. *Memoirs of the Museum of Comparative Zoology at Harvard College* 37: 1-243.

Boero F, Bouillon J (2005) Cnidaria and Ctenophora. *Marine parasitology.* CSIRO Publishing: 177-182.

Bouillon J (1978) Hydroméduses de la Mer de Bismarck (Papouasie, Nouvelle-Guinée). Partie 1: Anthomedusae Capitata (Hydrozoa - Cnidaria). *Cahiers de Biologie Marine* 19: 249-297.

Bouillon J (1984) *Sphaerocoryne peterseni*: nouvelle espèce d'anthoméduse de Papouasie Nouvelle-Guinée (Hydrozoa-Cnidaria). *Indo-Malayan Zoology* 1: 245-248.

Brinckmann-Voss A, Lindner A (2008) *Monocoryne colonialis* sp. nov., a colonial candelabrid hydroid (Cnidaria: Hydrozoa: Candelabridae) from the North Pacific. *Journal of the Marine Biological Association of the United Kingdom* 88: 1631-1635.

Calder D (1971) Hydroids and hydromedusae of southern Chesapeake Bay. *Virginia Institute of Marine Science. Special Papers in Marine Science* 1: 1–125

Calder D, Mallinson J, Collins K, Hickman C (2003) Additions to the hydroids (Cnidaria) of the Galápagos, with a list of species reported from the islands. *Journal of Natural History* 37: 1173-1218.

Calder DR (1988) Shallow-Water Hydroids of Bermuda: The Athecatae. *Royal Ontario Museum Life Sciences Contributions* 148: 1-107.

Calder DR (2010) Some anthoathecate hydroids and limnopolyps (Cnidaria, Hydrozoa) from the Hawaiian archipelago. *Zootaxa* 2590: 1-91.

Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution* 17: 540-552.

Conover JT, Sieburth JM (1964) Effect of *Sargassum* distribution on its epibiota and antibacterial activity. *Botanica Marina* 6: 147-157.

Cunha AF, Maronna MM, Marques AC (2016) Variability on microevolutionary and macroevolutionary scales: a review on patterns of morphological variation in Cnidaria Medusozoa. *Organisms Diversity & Evolution* 16: 431-442.

Cunningham CW, Buss LW (1993) Molecular evidence for multiple episodes of paedomorphosis in the family Hydractiniidae. *Biochemical Systematics and Ecology* 21: 57-69.

DiBattista JD, Howard Choat J, Gaither MR, Hobbs JPA, Lozano-Cortés DF, Myers RF et al., Westneat MW (2016) On the origin of endemic species in the Red Sea. *Journal of Biogeography* 43: 13-30.

Excoffier L, Lischer HE (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* 10: 564-567.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* 3: 294-299.

Fontana S, Keshavmurthy S, Hsieh HJ, Denis V, Kuo C-Y, Hsu C-M et al., Chen CA (2012) Molecular evidence shows low species diversity of coral-associated hydroids in *Acropora* corals. *PloS One* 7: e50130.

Fraser C (1938) Hydroids of the 1934 Allan Hancock Pacific Expedition. Hydroides de la Expedición al Pacífico de Allan Hancok en 1934. *Allan Hancock Pacific Expeditions.* 4: 1-105.

Galea HR (2008) On a collection of shallow-water hydroids (Cnidaria: Hydrozoa) from Guadeloupe and Les Saintes, French Lesser Antilles. *Zootaxa* 1878: 1-54.

Galea HR (2013) New additions to the shallow-water hydroids (Cnidaria: Hydrozoa) of the French Lesser Antilles: Martinique. *Zootaxa* 3686: 1-50.

Galea HR, Ferry R (2013) *Podocoryna martinicana*, a new species of athecate hydroid (Cnidaria: Hydrozoa: Hydractiniidae) from the Caribbean. *Zootaxa* 3710: 578-590.

Gili J-M, Hughes R (1995) The ecology of marine benthic hydroids. *Oceanography and Marine Biology* 33: 351-426.

Haeckel EHPA (1879) *Monographie der Medusen*. G. Fischer.

Hargitt CW (1904) Notes on a hitherto undescribed hydroid from Long Island Sound. *The Biological Bulletin* 7: 251-253.

Hargitt CW (1908) Notes on a few coelenterates of Woods Holl. *The Biological Bulletin* 14: 95-120.

Hirohito ES (1988) *The Hydroids of Sagami Bay*. Publications of the Biolological Laboratory, Imperial Household, Tokyo.

Hughes TP, Bellwood DR, Connolly SR (2002) Biodiversity hotspots, centres of endemicity, and the conservation of coral reefs. *Ecology Letters* 5: 775-784.

Katoh K, Standley DM (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution* 30: 772-780.

Lanfear R, Calcott B, Ho SY, Guindon S (2012) PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution* 29: 1695-1701.

Maggioni D, Montano S, Seveso D, Galli P (2016) Molecular evidence for cryptic species in *Pteroclava krempfi* (Hydrozoa, Cladocorynidae) living in association with alcyonaceans. *Systematics and Biodiversity* 14: 484-493.

McCrady J (1859) *Gymnopthalmata of Charleston harbor*. Russell and Jones.

Medlin L, Elwood HJ, Stickel S, Sogin ML (1988) The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene* 71: 491-499.

Mergner H, Wedler E (1977) Ueber die Hydropolypenfauna des Roten Meeres und seiner Ausgänge.“*Meteor” Forschungs-Ergebnisse* 24: 1-32.

Millard NAH (1975) Monograph on the Hydroida of Southern Africa. *Annales of the South African Museum* 68: 1-513.

Müller WA, Leitz T (2002) Metamorphosis in the Cnidaria. *Canadian Journal of Zoology* 80: 1755-1771.

Nagale P, Apte D (2014) Intertidal hydroids (Cnidaria: Hydrozoa: Hydroidolina) from the Gulf of Kutch, Gujarat, India. *Marine Biodiversity Records* 7: e116.

Nawrocki AM, Schuchert P, Cartwright P (2010) Phylogenetics and evolution of Capitata (Cnidaria: Hydrozoa), and the systematics of Corynidae. *Zoologica Scripta* 39: 290-304.

Orlov D (1997) The role of larval settling behaviour in determination of the specific habitat of the hydrozoan *Dynamena pumila* (L.). Larval settlement in *Dynamena pumila* (L.). *Journal of Experimental Marine Biology and Ecology* 208: 73-85.

Pearse A (1950) Notes on the inhabitants of certain sponges at Bimini. *Ecology* 31: 149-151.

Peña-Cantero ÁL, Sentandreu V (2017) Phylogenetic relationships of endemic Antarctic species of *Staurotheca* Allman, 1888 (Cnidaria, Hydrozoa). *Polar Biology* 40: 301-312.

Petersen KW (1990) Evolution and taxonomy in capitate hydroids and medusae (Cnidaria: Hydrozoa). *Zoological Journal of the Linnean Society* 100: 101-231.

Pictet C (1893) Étude sur les hydraires de la Baie d'Amboine. *Revue Suisse de Zoologie* 1: 1-64.

Piraino S, Todaro C, Geraci S, Boero F (1994) Ecology of the bivalve-inhabiting hydroid *Eugymnanthea inquilina* in the coastal sounds of Taranto (Ionian Sea, SE Italy). *Marine Biology* 118: 695-703.

Prévot E (1959) Morphologie et évolution des structures tentaculaires chez les hydraires gymnoblastes Capitata. *Recueil des Travaux de la Station Marine d’Endoume* 29: 91-128.

Puce S, Calcinai B, Bavestrello G, Cerrano C, Gravili C, Boero F (2005) Hydrozoa (Cnidaria) symbiotic with Porifera: a review. *Marine Ecology* 26: 73-81.

Puce S, Cerrano C, Di Camillo CG, Bavestrello G (2008) Hydroidomedusae (Cnidaria: Hydrozoa) symbiotic radiation. *Journal of the Marine Biological Association of the United Kingdom* 88: 1715-1721.

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S et al., Huelsenbeck JP (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* 61: 539-542.

Rützler K (1978) Sponges in coral reefs. *Coral Reefs: Research Methods. Monographs on Oceanographic Methodology*: 299-313.

Schuchert P (2010) The European athecate hydroids and their medusae (Hydrozoa, Cnidaria): Capitata part 2. *Revue Suisse de Zoologie* 117: 337-555.

Schuchert P, Reiswig HM (2006) *Brinckmannia hexactinellidophila*, n. gen., n. sp.: a hydroid living in tissues of glass sponges of the reefs, fjords, and seamounts of Pacific Canada and Alaska. *Canadian Journal of Zoology* 84: 564-572.

Schulze FE (1880) XXIV. On the Structure and arrangement of the soft parts in *Euplectella aspergillum*. *Earth and Environmental Science Transactions of the Royal Society of Edinburgh* 29: 661-673.

Shimabukuro V (2007) As associações epizóicas de Hydrozoa (Cnidaria: Leptothecata, Anthoathecata e Limnomedusae). Master's Thesis, Universidade de São Paulo.

Shimabukuro V, Marques AC, Migotto AE (2006) Fauna de hidrozoários atecados (Hydrozoa, Anthoathecata) da costa do Estado do Ceará, Brasil. *Biota Neotropica* 6: 1-13.

Sukumaran J, Holder MT (2010) DendroPy: a Python library for phylogenetic computing. *Bioinformatics* 26: 1569-1571.

Talavera G, Castresana J (2007) Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Systematic Biology* 56: 564-577.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution* 30: 2725-2729.

Tyler JC, Böhlke JE (1972) Records of sponge-dwelling fishes, primarily of the Caribbean. *Bulletin of Marine Science* 22: 601-642.

Uriz M-J, Rosell D, Maldonado M (1992) Parasitism, commensalism or mutualism? The case of Scyphozoa (Coronatae) and horny sponges. *Marine Ecology Progress Series* 81: 247-255.

Varela C (2012) Registros nuevos de hidrozoos (Cnidaria: Hydroidomedusae) para Cuba, con la descripción de una especie nueva. *Solenodon* 10: 1-7.

Vervoort W (1949) Notes on a small collection of hydroids from Jersey (Channel Islands). *Zoologische mededeelingen* 30: 133-162.

Warren E (1908) *On a collection of hydroids, mostly from the Natal coast*. Adlard and Son.

Wedler E, Larson R (1986) Athecate hydroids from Puerto Rico and the virgin Islands. *Studies on Neotropical Fauna and Environment* 21: 69-101.

Wulff JL (2006) Ecological interactions of marine sponges. *Canadian Journal of Zoology* 84: 146-166.

Yamada M, Konno K (1973) Polyp and medusa of the hydroid *Sphaerocoryne multitentaculata* (Warren) from Japan. *Publications of the Seto Marine Biological Laboratory* 20: 109-109.

Zietara MS, Arndt A, Geets A, Hellemans B, Volckaert FA (2000) The nuclear rDNA region of *Gyrodactylus arcuatus* and *G. branchicus* (Monogenea: Gyrodactylidae). *Journal of Parasitology* 86: 1368-1373.

Zwickl D (2006) GARLI: genetic algorithm for rapid likelihood inference. *See http://www. http://www.bio.utexas.edu/faculty/antisense/garli/Garli.html*.

4.7. TABLES

Table 4.1. Genetic distances (uncorrected p-distances in %) among and within sphaerocorynid species. Values are indicated as mean ± standard deviation. n.c. not calculated

A) *16S*	1	2	3	4	5
1) *S. agassizii*	3.2 ± 0.5				
2) *S. bedoti*	9.6 ± 1.2	1.2 ± 0.3			
3) *H. caribbensis*	8.9 ± 1.1	11.6 ± 1.3	0.6 ± 0.2		
4) *Sphaerocorynoides* sp.	5.6 ± 0.9	9.1 ± 1.2	7.8 ± 1.1	0.9 ± 0.2	
5) *A. cabela*	16.1 ± 1.6	18.1 ± 1.6	18.5 ± 1.6	15.4 ± 1.5	0.8 ± 0.3

B) *COX1*	1	2	3	4	5
1) *S. agassizii*	3.6 ± 0.4				
2) *S. bedoti*	16.9 ± 1.3	3.3 ± 0.4			
3) *H. caribbensis*	14.6 ± 1.3	17.7 ± 1.3	1.3 ± 0.3		
4) *Sphaerocorynoides* sp.	15.0 ± 1.3	17.8 ± 1.4	14.4 ± 1.3	0.5 ± 0.2	
5) *A. cabela*	n.c.	n.c.	n.c.	n.c.	n.c.

C) *COXIII*	1	2	3	4	5
1) *S. agassizii*	3.4 ± 0.5				
2) *S. bedoti*	17.8 ± 1.3	2.1 ± 0.3			
3) *H. caribbensis*	19.3 ± 1.4	20.3 ± 1.5	0.8 ± 0.2		
4) *Sphaerocorynoides* sp.	15.8 ± 1.3	17.5 ± 1.4	16.4 ± 1.4	0.9 ± 0.4	
5) *A. cabela*	23.7 ± 1.5	22.2 ± 1.5	23.0 ± 1.6	24.0 ± 1.6	3.5 ± 0.5

D) *18S*	1	2	3	4	5
1) *S. agassizii*	0.0 ± 0.0				
2) *S. bedoti*	0.7 ± 0.2	0.1 ± 0.0			
3) *H. caribbensis*	0.4 ± 0.2	0.8 ± 0.2	0.0 ± 0.0		
4) *Sphaerocorynoides* sp.	0.2 ± 0.1	0.6 ± 0.2	0.3 ± 0.1	0.0 ± 0.0	
5) *A. cabela*	2.0 ± 0.3	1.9 ± 0.3	2.1 ± 0.3	1.9 ± 0.3	0.0 ± 0.0

E) *28S*	1	2	3	4	5
1) *S. agassizii*	0.2 ± 0.1				
2) *S. bedoti*	2.0 ± 0.3	0.2 ± 0.0			
3) *H. caribbensis*	0.8 ± 0.2	1.6 ± 0.3	0.0 ± 0.0		
4) *Sphaerocorynoides* sp.	0.7 ± 0.2	1.7 ± 0.3	0.7 ± 0.2	0.1 ± 0.0	
5) *A. cabela*	3.4 ± 0.4	3.7 ± 0.5	3.2 ± 0.4	3.4 ± 0.4	0.0 ± 0.0

A) *ITS*	1	2	3	4	5
1) *S. agassizii*	2.1 ± 0.3				
2) *S. bedoti*	6.7 ± 0.9	0.5 ± 0.1			
3) *H. caribbensis*	3.0 ± 0.6	7.9 ± 1.0	0.1 ± 0.1		
4) *Sphaerocorynoides* sp.	3.0 ± 0.5	7.7 ± 1.1	2.9 ± 0.6	0.2 ± 0.1	
5) *A. cabela*	12.0 ± 1.1	15.2 ± 1.3	12.3 ± 1.2	11.3 ± 1.1	2.6 ± 0.8

4.8. FIGURES

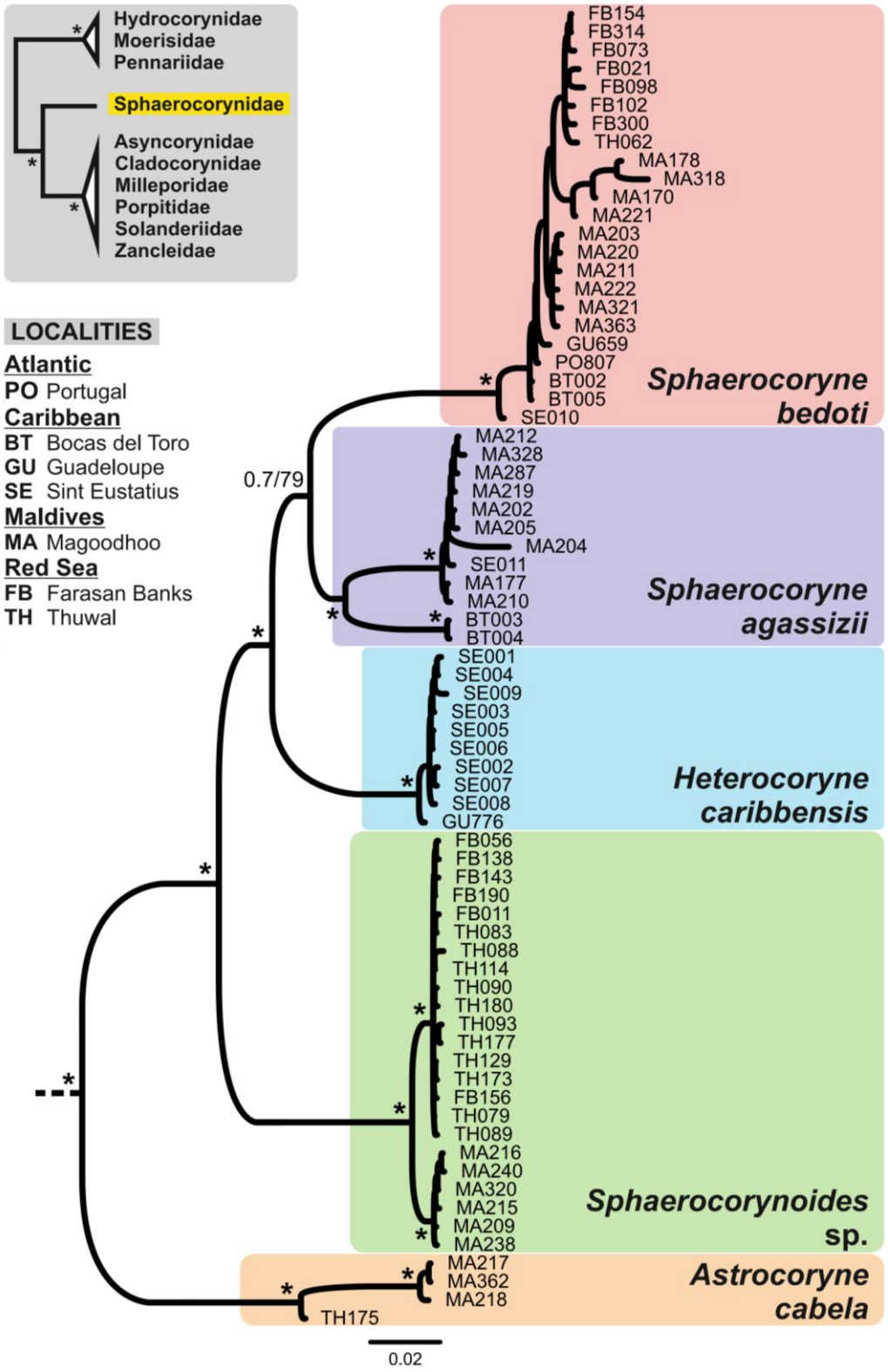

Figure 4.1. Bayesian phylogenetic hypothesis for the Sphaerocorynidae based on the concatenated mitochondrial and nuclear genes. Support at nodes is indicated as BPP/BS. Fully supported nodes (BPP > 0.95, BS > 95) are indicated with asterisks. Each species is highlighted with a different colour. Sampling localities are indicated for each specimen as coded in the legend. Position of the Sphaerocorynidae within the superfamily Zancleida is shown in the grey box.

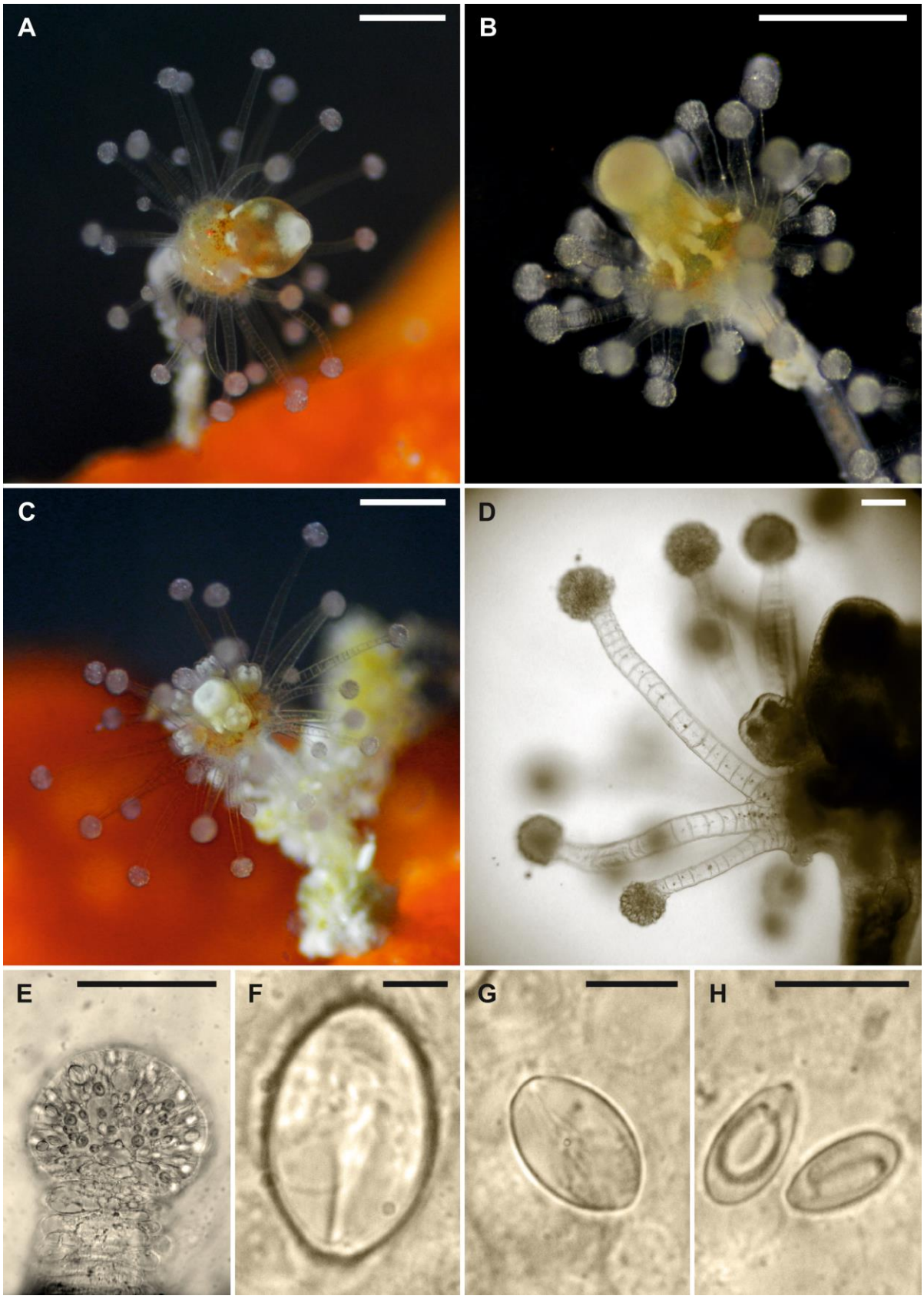

Figure 4.2. *Sphaerocoryne agassizii*. A, B) Polyp stages showing the typical colouration of the species. C) Fertile polyp with medusa buds carried singly on short blastostyles above the tentacles. D) Close-up of the polyp showing the longitudinal organisation of tentacles and a medusa bud. E) Capitulum of a tentacle with nematocysts inside, including F) large stenoteles, G) small stenoteles, and H) desmonemes. Scale bars: A-C) 0.5 mm, D) 0.1 mm E-G) 10 μm.

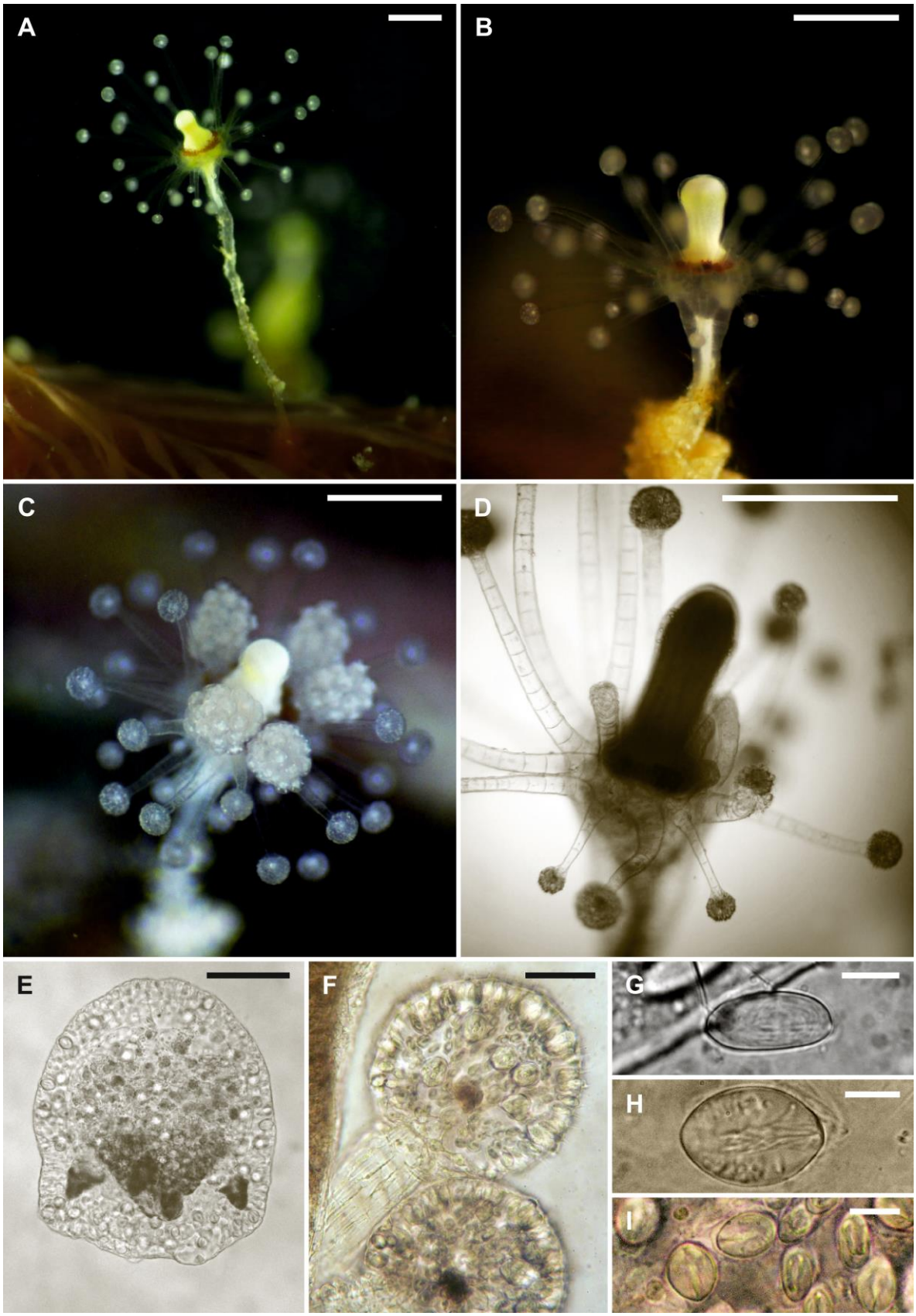

Figure 4.3. *Sphaerocoryne bedoti*. A, B) Polyp stages showing the typical colouration of the species. C) Fertile polyp with medusa buds carried in five large clusters on short blastostyles above the tentacles. D) Close-up of the polyp showing the tentacles not clearly organised. E) Newly liberated medusa. F) Capitula of a tentacle with light-reflecting inclusions in the middle (brown colour). G) Heteronemes, H) large stenoteles, and I) small stenoteles and desmonemes. Scale bars: A-D) 0.5 mm, E, F) 50 μm, G-I) 10 μm.

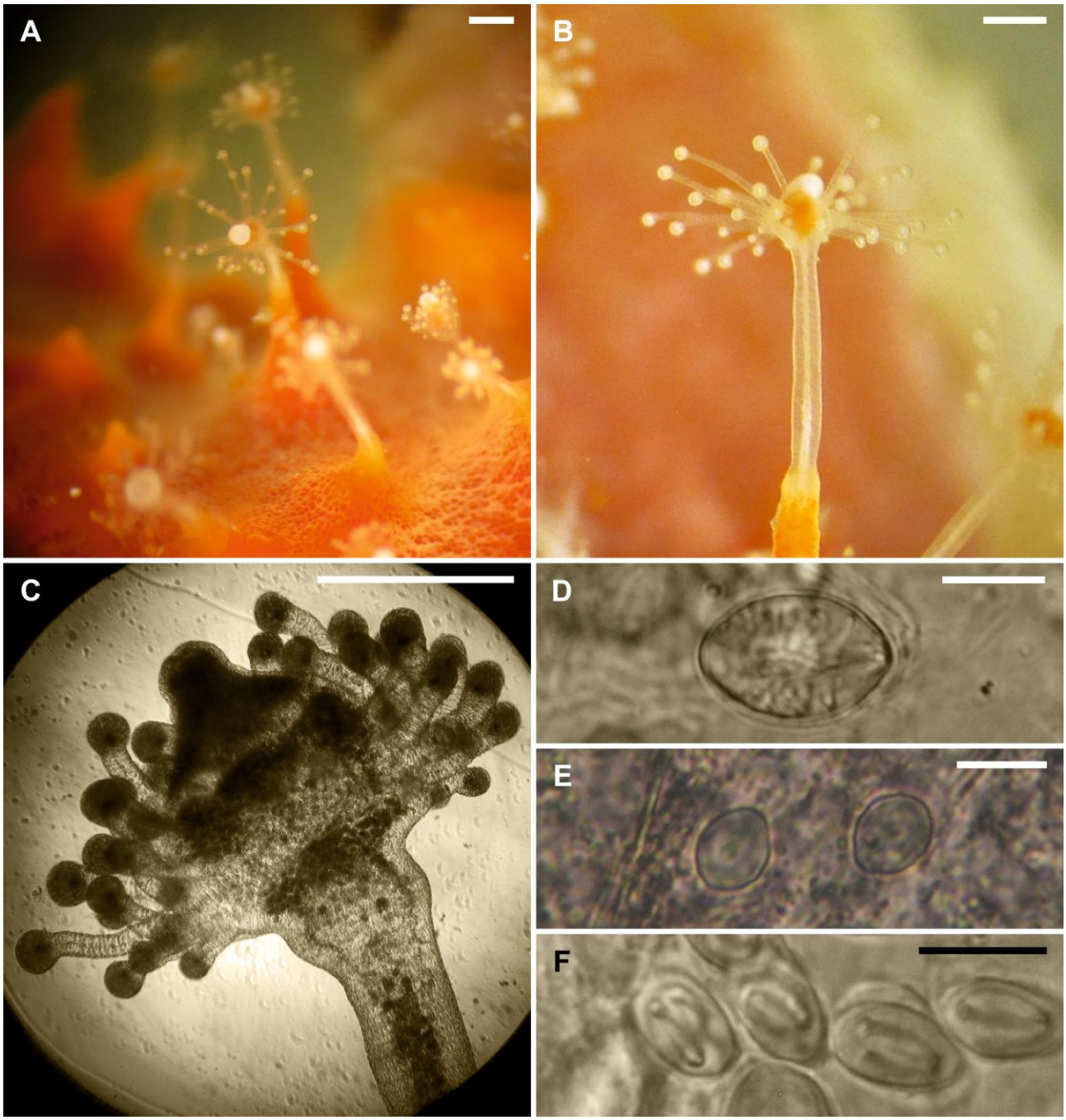

Figure 4.4. *Heterocoryne caribbensis*. A, B) Polyp stages associated with the sponge *Mycale* sp. C) Close-up of the polyp showing the typical organisation of tentacles, with partially fused proximal tentacles and with light-reflecting inclusions in the capitula. D) Large stenoteles, E) small stenoteles, and F) desmonemes. Scale bars: A-C) 0.5 mm, D-F) 10 μm.

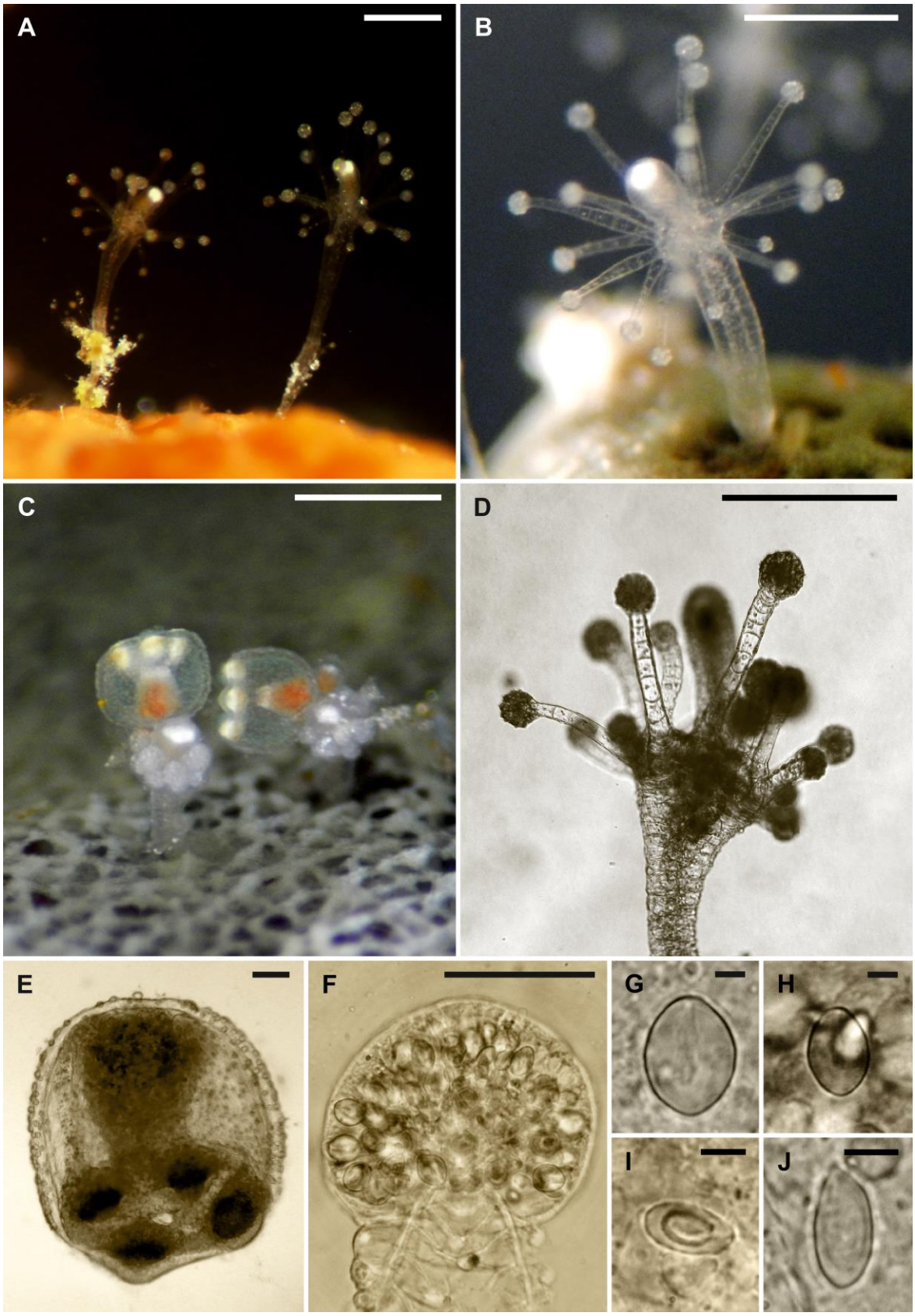

Figure 4.5. *Sphaerocorynoides* sp. A, B) Polyp stages showing the typically short pedicels. C) Fertile polyps carrying up to two medusa buds. D) Close-up of the polyps showing the tentacles organised in two close whorls. E) Newly released medusa. F) Capitulum of a tentacle with nematocysts inside, including G) large stenoteles, H) small stenoteles, I) desmonemes, and J) macrobasic mastigophores. Scale bars: A-D) 0.5 mm, E, F) 50 μm, G-J) 5 μm.

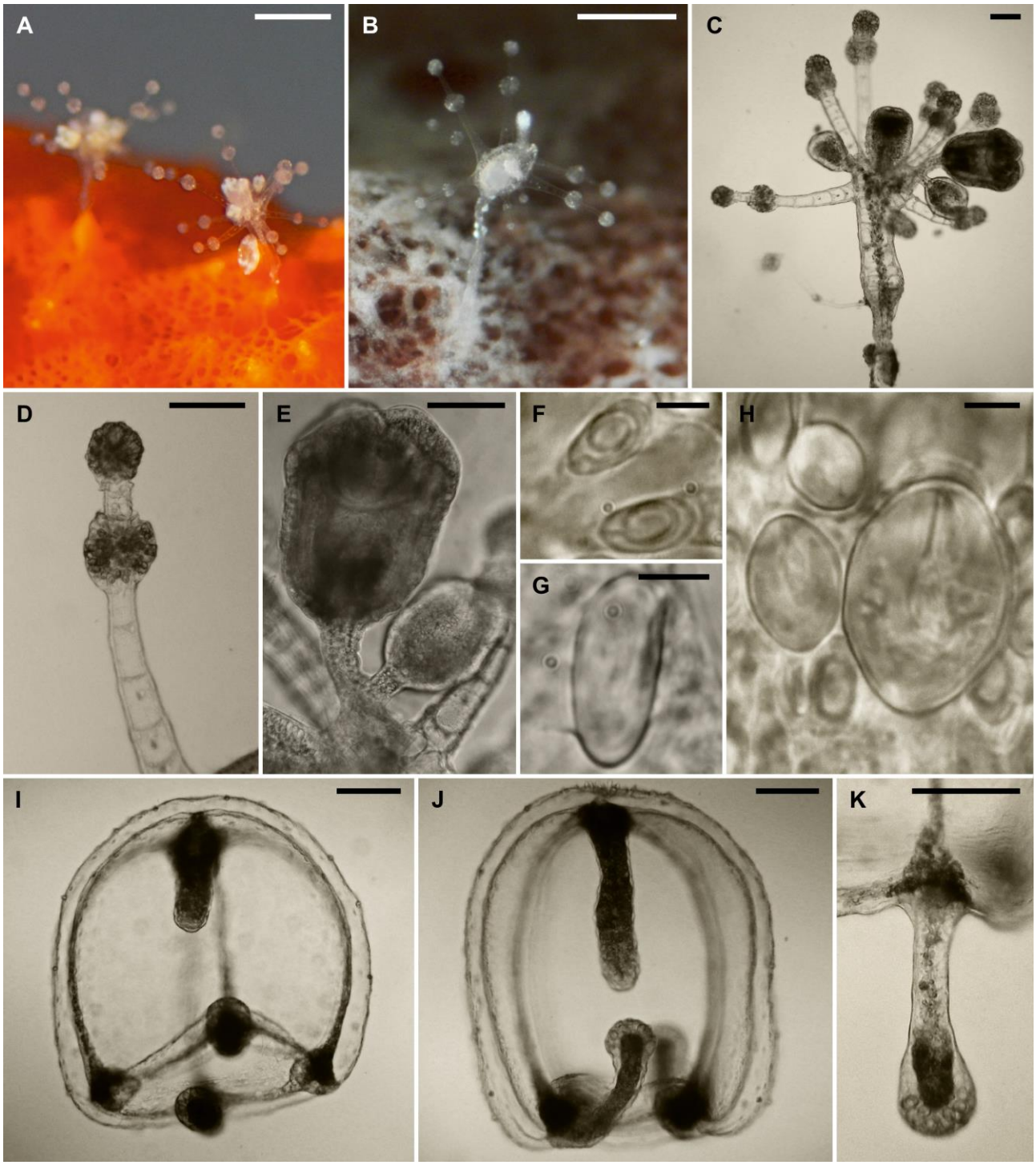

Figure 4.6. *Astrocoryne cabela*. A, B) Polyp stages from Maldives and Saudi Arabia, respectively. C) Fertile polyp with three medusa buds carried singly or in couple on blastostyles. D) Dicapitate tentacles, with a terminal capitulum and another proximal cluster of nematocysts. E) Two medusa buds at different stages of maturation. F) Desmonemes, G) microbasic euryteles, and H) stenoteles of three size classes. I, J) Newly liberated medusa and two day old medusa, respectively. K) Close-up of a medusa tentacle with a terminal cluster of nematocysts. Scale bars: A, B) 0.5 mm, C-E, I-K) 0.1 mm, F-H) 5 μm.

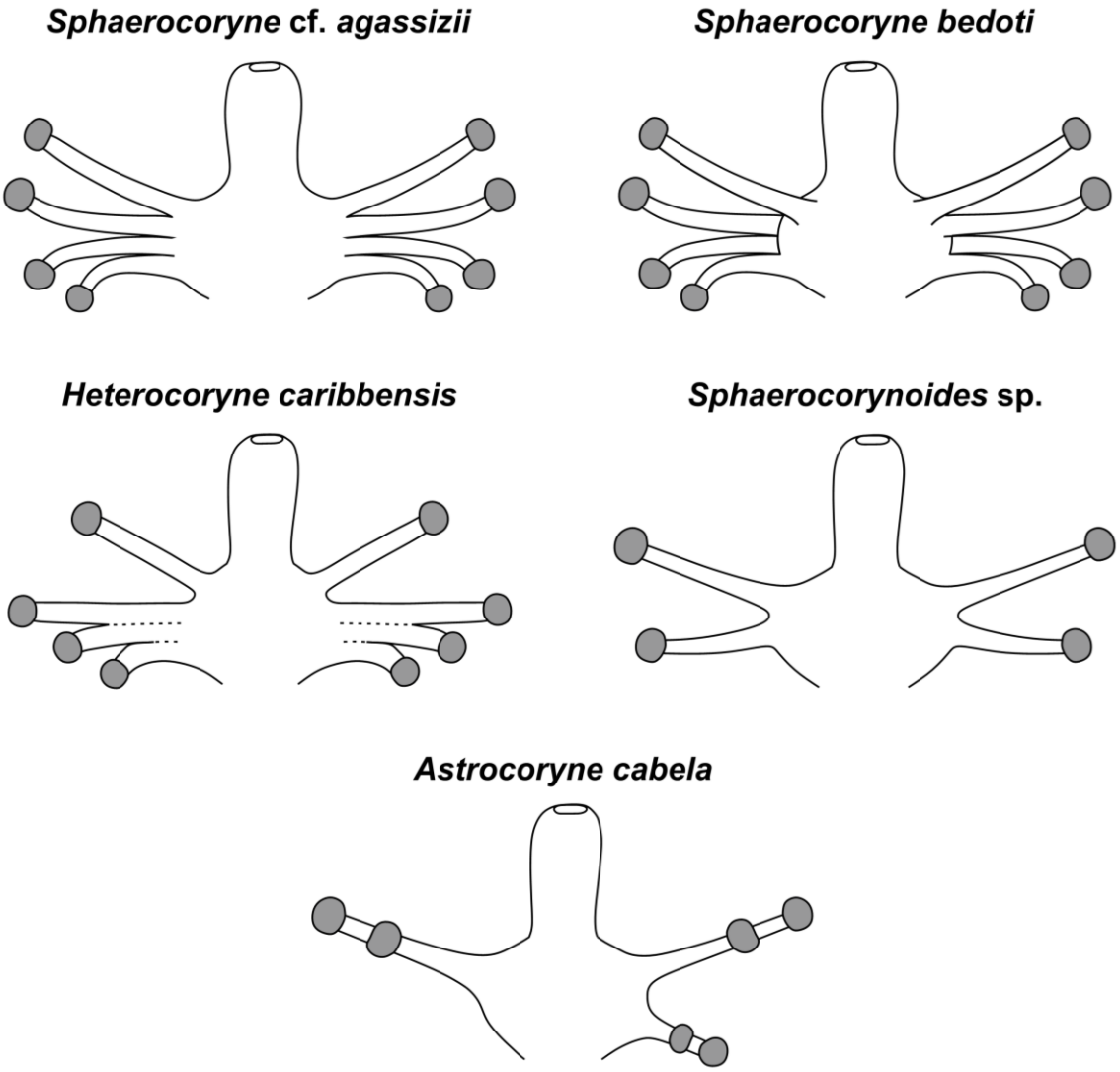

Figure 4.7. Tentacle arrangement in different sphaerocorynid species.

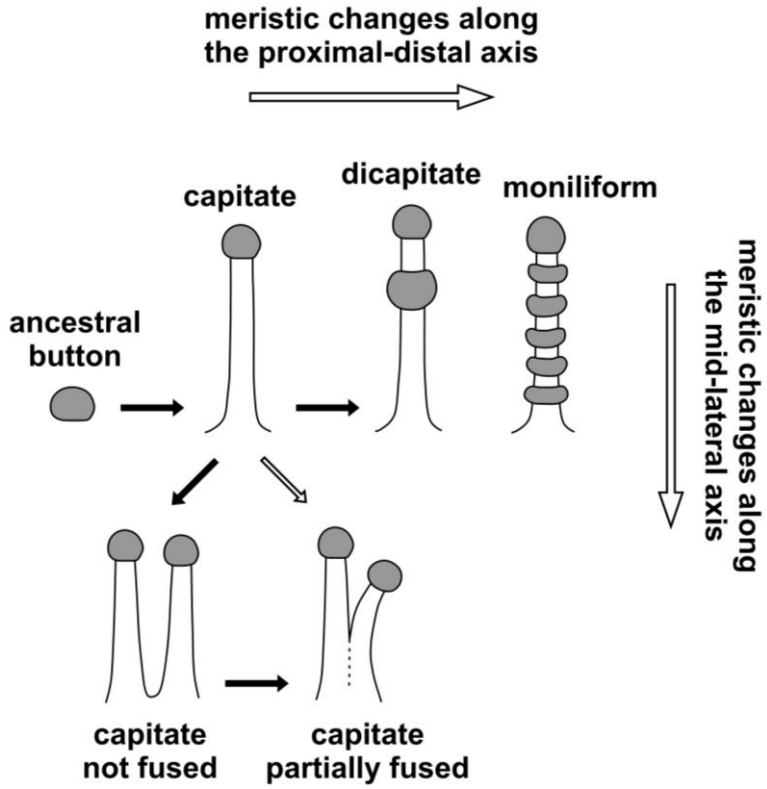

Figure 4.8. Different type of tentacles and their possible evolution. A) Primordial nematocyst button, B) capitate tentacle, C) dicapitate tentacle, D) moniliform tentacle, E) capitate tentacles in multiple whorls, F) partially fused capitate tentacles that may have derived from partial duplication (white arrow) or partial fusion (black arrow) events.

CHAPTER 5

Molecular phylogeny and evolution of the superfamily Zancleida (Hydrozoa, Capitata), with a focus on the polyphyletic family Zancleidae

This work is in preparation for the submission as:

1. Maggioni D, Galli P, Seveso D, Berumen ML, Arrigoni R, Montano S. Molecular phylogeny and evolution of the superfamily Zancleida (Hydrozoa, Capitata), with a focus on the polyphyletic family Zancleidae.

5.1. ABSTRACT

The superfamily Zancleida is a diverse group of capitate hydrozoans, and includes species showing a wide range of morphological and ecological features as well as many symbiotic taxa. The taxonomy and systematics of many species is still far to be elucidated due to the presence of few suitable diagnostic morphological characters in closely related species, the lack of information on complete life cycles, and the undersampling in molecular works. This is especially true for the family Zancleidae, which has already been demonstrated to harbour a cryptic diversity, due to the conserved morphology of both polyps and medusae. Here, the most comprehensive phylogenetic assessment of the Zancleida is presented, with a focus on the family Zancleidae. This latter group is here proven to be polyphyletic both at family and genus level and to hide a further diversity, due to the presence of cryptic species. The study of the evolution of selected morphological, ecological and reproductive characters did not allow a clear reconstruction of previously hypothesised evolutionary trends in symbiotic species, neither for the Zancleidae nor for the Zancleida. These analyses rather showed a certain level of convergence and confirmed the difficulty in the delineation of clear diagnoses for the genera and species belonging to the Zancleidae, highlighting the importance of further morphological, molecular, and ecological assessments.

5.2. INTRODUCTION

The capitate hydrozoans (Hydrozoa, Capitata) belong to a suborder in the polyphyletic order Anthoathecata, and are divided in the two superfamilies Zancleida and Corynida (Nawrocki et al. 2010). Traditionally, also representative of the clade now known as Aplanulata were included in the Capitata, but molecular and life history analyses clearly demonstrated that the Aplanulata constitute an independent evolutionary lineage (Collins et al. 2005, Kayal et al. 2015). Within the Capitata, the superfamily Zancleida shows a diverse array of morphological, reproductive, ecological, and behavioural traits. For instance many species have maintained a *Zanclea*-like aspect of the polyps, whereas other have evolved peculiar traits, including different type of tentacles, sets of nematocysts, and colony organisation (Petersen 1990, Bouillon et al. 2006). Most of the species reproduce via the medusa stage, but in some cases also other more derived structures are found, such as eumedusoids and cryptomedusoids (Bouillon et al. 2006). A certain variety can be observed also in the ecological preferences of the Zancleida. For instance, in this group are included both marine and freshwater species, temperate, tropical and polar species, generalist and symbiotic species, and invasive species (Stepanjants 1972, Jankowski 2001, Bouillon et al. 2006, Puce et al. 2008, Miglietta et al. 2015). In some cases, certain species have developed typical behaviours, such as *Halocoryne epizoica* Hadzi 1917 that specifically feeds on the lophophoral tentacles of its bryozoan host (Piraino et al. 1992), and *Samuraia tabularasa* Mangin 1991 that is able to maintain the surrounding rock substrate free of barnacles (Mangin 1991).

The evolutionary relationships within the two well-supported clades of the Capitata are still not fully resolved, mostly due to the limited sampling of many groups, the few DNA sequences available, the marker resolution, and the lack of clear morphological synapomorphies. Indeed, the most comprehensive molecular study available to date left some doubts about the relationships among different families and genera, even if in some cases the phylogenetic resolution was strong enough to take taxonomic decisions (Nawrocki et al. 2010). The two most challenging groups within the Capitata are the families Zancleidae Russel 1953 and Corynidae Johnston 1836, due to the morphological simplicity, the often not fully known life cycles, the species diversity, and the taxonomic confusion that characterise many species (Schuchert 2001, Schuchert 2010). In particular, the Zancleidae comprehends three genera, namely *Zanclea* Gegenbaur 1856, *Zanclella* Boero & Hewitt 1992, and *Halocoryne* Hadzi 1917 (Boero et al. 2000), even if some authors argued against the separation of these three taxa based on morphological characters alone (Schuchert 2010). As a matter of fact, the characters used to discriminate among the three genera have often overlapping states. For instance, *Zanclella* is separated from *Zanclea* due to ‘gastrozooid usually with reduced number of tentacles’ and ‘umbrella laterally compressed in the tentacular plane’ (Bouillon et al. 2006), but the first character is too gradual to be applied reliably (for instance *Zanclella diabolica* Boero, Bouillon & Gravili 2000 has gastrozooids highly resembling *Zanclea* (Boero et al. 2000)) (Schuchert 2010) and the type species *Zanclella bryozoophila* Boero & Hewitt 1992 has a medusa not laterally compressed (Boero and Hewitt 1992). Furthermore, as highlighted by Schuchert (2010), an evaluation of the relationships of *Halocoryne* species with other Zancleidae is made difficult by the

reduction of both polyp and medusa stages and only a proper molecular phylogeny of the three genera may help resolving this issue.

The family Zancleidae has a worldwide distribution, with most of the species found in tropical and subtropical waters (Kramp 1968, Ristedt and Schuhmacher 1985, Calder 1988), but some can be found also in temperate seas (Gravili et al. 1996) and in the Southern Ocean (Stepanjants 1972), from shallow waters (Boero et al. 2000, Pantos and Bythell 2010) up to 500 m deep (Bouillon et al. 2000). Of all 40 nominal species ascribed to this family (34 *Zanclea*, 3 *Zanclella*, and 4 *Halocoryne*), a dozen have been described exclusively based on medusa specimens (Haeckel 1879, Browne 1916, Kramp 1959, Uchida and Sugiura 1976, Xu et al. 1991, Gershwin and Zeidler 2003, Xu et al. 2008), whereas six are based on the polyp stage only (Agassiz 1862, Stepanjants 1972, Boero et al. 2000, Puce et al. 2002, Galea 2008, Varela 2012). Among the species with a known polyp stage, most prefer living substrates, usually forming symbiotic relationships with marine organisms such as bivalves, bryozoans, algae, octocorals, and scleractinians. Some species can be found living on both biotic or abiotic substrates, such as *Zanclea implexa* (Alder 1856) and *Zanclea giancarloi* Boero, Bouillon & Gravili 2000 (Schuchert 2010), but in most cases the associations appear to be obligate at least for the hydroid, such as for *Zanclella, Halocoryne* and ten *Zanclea* species associated with bryozoans (Boero et al. 2000), seven *Zanclea* species associated with scleractinians (Maggioni et al. in preparation), *Zanclea costata* Gegenbaur 1857 associated with bivalves, and *Zanclea alba* (Meyen 1834) living on free-floating algae (Boero et al. 2000). Different species show variable degrees of integration with their hosts. A common feature in *Zanclella*, *Halocoryne*, and some *Zanclea* species is the reduction of the number and size of tentacles, likely due to the fact that polyps can exploit the water movement generated by the host and have developed specific trophic strategies, the presence of a specialised hydrorhiza and the polymorphism (Puce et al. 2007). It has been hypothesised that the reduction and modification of both polyp and medusa stages, the development of peculiar host-related behaviours, along with an evolutionary trend in the protection of the hydrorhiza may reflect the level of integration between the hydroid and its host (Boero et al. 2000, Puce et al. 2002). Some *Zanclea* species have a perisarc-covered hydrorhiza crawling on the substrate: *Z. alba* and *Zanclea migottoi* Galea 2008 on algae (Calder 1988, Galea 2008); *Z. costata* and *Zanclea fanella* Boero, Bouillon & Gravili 2000 on bivalves (Boero et al. 2000); and *Z. implexa*, *Z. giancarloi*, *Zanclea hicksoni* (Stepanjants 1972) on various substrates (Boero et al. 2000, Schuchert 2010, Peña Cantero et al. 2013). Other *Zanclea* species have their perisarc-covered hydrorhiza growing under the tissues of the host and under or overgrown by the skeleton of the host: *Zanclea sango* I, II, and *Zanclea intermedia* I, II, III associated with corals (Maggioni et al. In preparation); *Zanclea sessilis* (Gosse 1853) and *Zanclea tipis* Puce, Cerrano, Boyer, Ferretti & Bavestrello 2002 associated with bryozoans (Boero et al. 2000, Puce et al. 2002); and *Z. giancarloi* when found in association with bryozoans (Boero et al. 2000). Many symbiotic *Zanclea*, and all *Zanclella* and *Halocoryne* species have a naked hydrorhiza extending inside the skeleton or under the tissues of their hosts: *Zanclea gallii* I, II, *Zanclea gilii* Boero, Bouillon & Gravili 2000 and *Zanclea margaritae* Pantos & Bythell 2010 associated with scleractinians (Boero et al. 2000, Pantos and Bythell 2010, Maggioni et al. In preparation); *Zanclea bomala* Boero, Bouillon & Gravili 2000, *Zanclea divergens* Boero, Bouillon & Gravili 2000, *Zanclea*

eilatensis Pica, Bastari & Puce 2017, *Zanclea hirohitoi* Boero, Bouillon & Gravili 2000, *Zanclea polymorpha* Schuchert 1996, *Zanclea protecta* Hastings 1932, *Zanclea retractilis* Boero, Bouillon & Gravili 2000, *H. epizoica, Halocoryne pirainoid* Boero, Bouillon & Gravili 2000, *Halocoryne frasca* Boero, Bouillon & Gravili 2000, *Z. bryozoophyla, Z. diabolica,* and *Zanclella glomboides* Boero, Bouillon & Gravili 2000 associated with bryozoans (Boero et al. 2000, Pica et al. 2017); *Zanclea cubensis* associated with octocorals (Varela 2012). Finally, the only species having a naked hydrorhiza crawling on the surface of the host is *Zanclea exposita* Puce, Cerrano, Boyer, Ferretti & Bavestrello 2002 associated with bryozoans (Puce et al. 2002). Given these characteristics in the organisation of the hydrorhiza, Puce et al. (2002) hypothesised that primitive species of *Zanclea* were epibiontic, living on various substrates and producing a hydrorhiza covered by perisarc. Subsequently, the specific association with bryozoans and the protection provided by the skeleton of the host promoted a progressive elimination of the perisarc and the bryozoan-associated perisarc-free genera *Zanclella* and *Halocoryne* may have derived from these species. The authors also assumed that this trend could be linked to the variation of the position of the medusa buds, since 'more primitive' species with exposed perisarc-covered hydrorhiza have buds among proximal tentacles, whereas 'more derived' species with naked hydrorhiza embedded in the host have buds growing at the base of hydranths and from the hydrorhiza. Another trend, noted by Boero et al. (2000), consists in the evolution of polymorphic colonies. The authors intended the presence of a hydrorhiza armed with nematocyst batteries in *Z. divergens* as a first step towards this complication of the colony. A further tendency towards polymorphism is then seen in the reduced gastrozooids of *Z. bomala,* which can be interpreted as dactylozooids. Finally, the complete polymorphism is reached in *Z. gilii, Z. hirohitoi* and *Z. polymorpha* with the presence of proper dactylozooids or tentaculozooids. The latter species were included in the 'polymorpha' group, whereas the monomorphic species were grouped in the 'alba' group.

Most of the authors that studied the Zancleidae highlighted the complicated evolutionary history and the difficulty in delineating the limits of the genera and species of this group. Moreover, molecular studies showed the non monophyly of the genus *Zanclea,* with *Zanclea prolifera* Uchida & Sugiura 1976 more closely related to *Asyncoryne ryniensis* Warren 1908 than to *Z. sessilis*, *Z. giancarloi*, and *Z. costata* (Nawrocki et al. 2010, Schuchert 2010). The aim of this study is therefore to shed light on the evolution and relationships of zancleid hydrozoans and more generally of the whole superfamily Zancleida. To do that, several species of the Zancleidae, along with other representative of the Zancleida, were collected from different localities and used to build a phylogenetic hypothesis and to study the possible evolution of important morphological and ecological traits.

5.3. MATERIALS AND METHODS

5.3.1. Sample Collection and Morphological Analyses

The sampling was conducted between March 2014 and May 2017 in several localities including the Indian Ocean, the Pacific Ocean, The Red Sea, the Caribbean Sea, and the Mediterranean Sea.

Representatives of the Zancleida were collected using hammer and chisel or a knife during snorkelling or SCUBA diving surveys. 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 placed under a stereomicroscope. Afterwards, they were immediately preserved in 95 % ethanol for molecular analyses and in 10 % formalin for morphological studies. Additional portions of the colonies were cultured in small bowls through feeding *Artemia* nauplii to the polyps to observe the medusa release. The medusae were then maintained in small bowls at ambient temperature and cultivated for some days. The water was replaced daily, two hours after feeding. The reared medusae were observed every day, and certain medusae were fixed in 10% formalin. Morphological observations and measurements of the polyps, medusae and nematocysts were mainly performed using living specimens in order to identify each specimen at species level. Underwater photographs of hydrozoans were taken using a Canon G11 camera in a Canon WP-DC 34 underwater housing. Microphotographs of hydroids, medusae and nematocysts were taken using a Leica EZ4 D stereomicroscope and a Zeiss Axioskop 40 microscope both equipped with a Nikon AW 100 camera and ocular micrometrics. Additional DNA material was obtained from the Natural History Museum of Geneva and was included in the molecular analyses.

5.3.2. Molecular and Evolutionary Analyses

The total genomic DNA of 21 ethanol-fixed Zancleidae species, 24 other Zancleida species and three outgroups was extracted following a protocol modified from Zietara et al. (2000). Six different molecular markers were amplified: i) a ~600 bp portion of the mitochondrial *16S* ribosomal DNA gene (*16S*), ii) a ~700 bp portion of the mitochondrial cytochrome oxidase subunit I gene (*COX1*), iii) a ~700 bp portion of the mitochondrial cytochrome oxidase subunit III gene (*COX3*), iv) a ~1700 bp portion of the nuclear *18S* ribosomal DNA gene (*18S*), v) a ~1700 bp portion of the nuclear *28S* ribosomal DNA gene (*28S*), and vi) a ~700 bp portion of the nuclear internal transcribed spacer ribosomal region (*ITS*). *16S*, *COX3*, *28S*, and *ITS* regions were amplified using hydrozoan-specific primers and protocols (Cunningham and Buss 1993, Fontana et al. 2012, Maggioni et al. 2016, Peña-Cantero and Sentandreu 2017), whereas *COX1* and *18S* genes were amplified using universal primers and protocols (Medlin et al. 1988, Folmer et al. 1994). All PCR products were purified with Illustra ExoStar (GE Healthcare) at 37° for 60 min, followed by 85° for 15 min and then directly sequenced in forward and reverse directions using an ABI 3730xl DNA Analyzer (Applied Biosystems). The obtained chromatograms were visually checked and assembled using Sequencher 4.1.4 (Gene Codes). *COX1* and *COX3* sequences were translated in Geneious 6.1.6 (Drummond et al. 2010), in order to check for the presence of stop codons. The obtained sequences of each marker plus other sequences retrieved from GenBank were aligned with MAFFT v. 7.110 (Katoh and Standley 2013) using the E-INS-i option and *16S*, *18S*, *28S*, and *ITS* alignment were run through Gblocks (Castresana 2000, Talavera and Castresana 2007) using the default 'less stringent' settings in order to remove ambiguously aligned regions. The sequences were concatenated in two dataset: a first one including all the available and generated sequences of the Zancleidae and closely related species; a second one including only one representative for each species of the Zancleida. PartitionFinder 1.1.1

(Lanfear et al. 2012) was used to determine the partition scheme and the molecular models under the Akaike Information Criterion (AIC). Bayesian inference (BI) and maximum likelihood (ML) were used to infer phylogenetic relationships of the two datasets. BI analyses were performed using MrBayes 3.2 (Ronquist et al. 2012). Four parallel Markov Chain Monte Carlo runs (MCMC) were run for 10^7 generations. Trees were sampled every 100^{th} generation, and burn-in was set to 25%. ML trees were built with Garli 2.01 (Zwickl 2006) and read into the SumTrees 4.0.0 program in the DendroPy 4.0.0 package (Sukumaran and Holder 2010) to calculate non-parametric bootstrap support values from 1000 replicates, each based on five heuristic search replicates, and to map them on the best ML tree. Bayesian posterior probabilities (BPP) and bootstrap values (BS) were indicated at each node. The *16S rRNA* pairwise genetic distances (uncorrected p-distance, 1000 bootstrap replicates) were also estimated using MEGA 6 (Tamura et al. 2013).

The evolution of six characters was studied by mapping their transformation pathways along the BI tree of the Zancleida, using the maximum likelihood approach and applying the MK1 model of Lewis (2001) with Mesquite 2.75 (Maddison and Maddison 2001). The following characters and states were considered. i) Association with other organsims: 0, bryozoans; 1, scleractinians; 2, bivalves; 3, algae; 4, octocorals; 5, sponges; 6, generalist (associated with various organisms); 7, not associated. ii) Polymorphism (presence of dactylozooids): 0, present; 1, absent. iii) Organisation of the hydrorhiza: 0, perisarc-covered and crawling on the surface of the substrate; 1, perisarc-covered and crawling under the skeleton or tissues of the host; 2, naked and crawling under the skeleton or tissues of the host; 3, other (colonies pelagic, calcifying, or with a chitinous endoskeleton). iv) Position of medusa buds on polyps: 0, in the proximal half and from the stolon or from the stolon; 1, in the proximal half; 2, in the oral half; 3, other (ampullae of milleporids, medusoids of solanderiids, or medusae of porpitids). v) Reproductive structures: 0, free-swimming medusa; 1, free-swimming eumedusoid; 2, fixed eumedusoid or cryptomedusoids. vi) Organisation of the colony: 0, encrusting; 1, upright (including also the colony type of milleporiids and solanderiids); 2, pelagic; 3, solitary.

5.4. RESULTS

Throughout the field surveys, 70 colonies of Zancleidae were collected in the investigated localities (Figures 5.1, 5.2). Most of the samples were identified at species level, but in some cases, the identification was not possible due to the incomplete information or the bad preservation of the colony. For the same reasons, some of the DNA extracts received for the Natural History Museum of Geneva were identified only at genus level. After the Gblock treatment, the total alignments of the *16S*, *COX1*, *COX3*, *18S*, *28S*, and *ITS* datasets were 575, 607, 608, 1606, 1609, and 453 bp long, respectively, and the concatenated dataset was 5518 bp long. PartitionFinder found the following partition scheme and models for the concatenated dataset (AIC): *16S* *COX3*_pos3 (GTR+ G + I), *COX1*_pos1 (F81 + G), *COX1*_pos2 (GTR+ G + I), *COX1*_pos3 (GTR + G), *COX3*_pos1 *ITS* (HKY + G + I), *COX3*_pos2 (HKY + G), *18S* (GTR + G + I), *28S* (K80 + I + G). Phylogenetic trees obtained from BI and ML analyses were highly similar and only the Bayesian topologies are shown in Figure 5.3 and 5.4. According to the obtained phylogenetic hypothesis, the family Zancleidae is polyphyletic

due to the clustering of some species with representatives of Asyncorynidae Kramp 1949, Milleporidae Fleming 1828 and Solanderiidae Marshall 1892 (Figure 5.3). Specifically, the Zancleidae can be subdivided in three monophyletic groups, here called Zancleidae 1, 2, and 3. The first group comprehends most of the *Zanclea* species and one of the two *Halocoryne* species (*Halocoryne pirainoid*) included in the analysis. The monophyly of this group, as well as the internal nodes, are in almost all cases fully supported and since this clade includes the type species *Zanclea costata*, it should be intended as the 'real' Zancleidae family and *Zanclea* genus. Other than the type species, the Zancleidae 1 is composed of *Zanclea sessilis, Zanclea giancarloi, Zanclea alba, Zanclea implexa,* all coral-associated *Zanclea* and unidentified *Zanclea* species from Maldives, China Sea, and Mediterranean Sea. Moreover, the clade *Zanclea* 1 is paraphyletic in relation to *H. pirainoid* (*Halocoryne* 1), which is here located as the sister species of coral-associated *Zanclea*. The clade called Zancleidae 2 includes only the species *Zanclea prolifera* (*Zanclea* 2) and is the sister group of *Asyncoryne ryniensis* (family Asyncorynidae), even if this relationship is poorly supported. A third clade of Zancleidae (Zancleidae 3) is the sister group of the reciprocally monophyletic families Milleporidae and Solanderiidae. In Zancleidae 3 representative of the three genera *Zanclea*, *Halocoryne* and *Zanclella* species are included. The genera *Zanclella* and *Halocoryne* are monophyletic within this clade, whereas *Zanclea* is polyphyletic. In particular, *Zanclea eilatensis* and *Zanclea protecta* (*Zanclea* 3) are sister species and are more closely related to *Halocoryne epizoica* (*Halocoryne* 2) than to the other species. Contrarily, *Zanclea divergens* (*Zanclea* 4) is sister of the genus *Zanclella*, which includes in this analysis three species, namely *Zanclella diabolica*, *Zanclella labiata* sp. nov. (see 'Taxonomic Section'), and *Zanclella* sp. from the Red Sea. Moreover, *Z. divergens* show a high degree of intra-specific variability (*16S rRNA* mean intra-specific genetic distance: 5.3 ± 0.6 %) and four main molecular lineages are recovered in the phylogeny (two from Maldives, one from Indonesia and one from Saudi Arabia). The *16S rRNA* genetic distances among these clades are high, ranging from 6.3 ± 1.1 to 10.5 ± 1.4 %. (Table 5.1). Generally, even if according to molecular analyses it is likely that different new species are present in the Zancleidae dataset, a formal description was possible only for one species (see 'Taxonomic Section'). Concerning the phylogenetic hypothesis of the whole Zancleida (Figure 5.4), with the exception of the Zancleidae and the Moerisiidae Poche 1914, all other families and genera are monophyletic. Specifically, the Porpitidae Goldfuss 1818 are sister of the groups described above, followed by the Cladocorynidae Allman 1872, the Sphaerocorynidae Prévot 1959 and the clade composed of the Pennariidae McCrady 1859, Hydrocorynidae Rees 1957, Halimedusidae Arai & Brinckmann-Voss 1980, and Moerisidae. The Moerisiidae is here paraphyletic due to the sister relationship between *Odessia maeotica* (Ostroumoff 1896) and *Tiaricodon coeruleus* Browne 1902.

The mapping of the studied characters on the phylogenetic tree of the Zancleida indicates that, in most cases, the observed states have occurred independently in different not-monophyletic lineages (Figures 5.5, 5.6, 5.7). The symbiotic lifestyle has occurred multiple times independently in the Zancleida (Figure 5.5A). The association with octocorals probably emerged in the most recent common ancestor of *Pteroclava* Weill 1931 and *Pseudozanclea*, whereas the symbiosis with sponges is likely to have appeared in the ancestor of the Sphaerocorynidae. Similarly, the association with scleractinians was gained in the most common ancestor of

the *Zanclea gallii*, *Zanclea sango*, and *Zanclea intermedia* complexes. Contrarily, the association with bryozoans emerged independently in the Zancleidae 1 (two times) and 3 (one time). The highest diversity in hosts can be observed within the Zancleidae 1, and this makes difficult to understand what was likely to be the ancestral host(s) of this group. The polymorphism evolved independently in multiple lineages, and originated different structures (Figure 5.5B). Polymorphic colonies are found in the pelagic Porpitidae, as well as in Milleporidae and in some representatives of the Zancleidae 1 and 3. The hydrorhiza can be covered or not by a perisarc and, in symbiotic species, can crawl both on the surface and inside the host. These states are more or less randomly distributed in the tree and do not seem to follow a clear evolutionary trend (Figure 5.6A). However, in all species associated with bryozoans, scleractinians, octocorals and sponges, the hydrorhiza grown embedded by the skeleton or tissues of the host. The perisarc is lost in all Zancleidae 3, and independently in the *Z. gallii* complex, in *H. pirainoid* and in *Pseudozanclea timida*. The position of medusa buds varies independently from the level of specialisation of the hydrorhiza (Figure 5.6B). For instance, medusa buds are carried on blastostyles directly arising from the hydrorhiza in species with both perisarc-covered and naked hydrorhiza. However, among symbiotic species, medusa buds are borne in the oral half only in sponge-associated species (i.e. Sphaerocorynidae). Regarding the reproductive structures, almost all species produce free-swimming medusae (Figure 5.7A), and a reduction in the medusa stage emerged multiple times. For instance, a reduction leading to cryptomedusoids and free eumedusoids is observed in the reciprocally monophyletic Solanderiidae and Milleporidae, respectively. Also *Pennaria* Goldfuss 1820 shows a reduced swimming medusoid, whereas the genera *Cladocoryne* Rotch 1871 and *Heterocoryne* Wedler & Larson 1986 underwent independent reductions leading to fixed medusoids. Finally, the growth form of the colony in the Zancleida is encrusting for the majority of the taxa (Figure 5.7B), but Solanderiidae and Milleporidae developed upright and in some cases massive colonies. *Pennaria* is the only other taxon to show upright and moderately big colonies, whereas a trend to solitary polyps emerged in the Moerisiidae and Halimedusidae.

5.4.1. Taxonomic Section

In the following paragraph, some of the collected and examined species are described in order to provide information about the newly described species and the species for which an identification at species level was not possible.

***Zanclella labiata* sp. nov.**

Figure 5.8

<u>*Polyp*</u>: Colony stolonal, growing in association with bryozoans (Figure 5.8A). Hydrorhiza naked, reticular, crawling under the bryozoan skeleton, often projecting out at the corners of zooeciae for some of its length (Figure 5.8B). Hydrorhiza filled with macrobasic holotrichous euryteles (Figure 5.8D, E). Gastrozooid cylindrical, up to 1.2 mm long, with an apical circular mouth, and with four oral tentacles and 20-30 aboral

tentacles scattered over the distal 3/4 of the polyp (Figure 5.8A). Tentacles short, with terminal capitations filled with nematocysts (stenoteles of two size classes) and with light-reflecting inclusions (Figure 5.8C), oral tentacles with bigger capitula (90-100 μm) and aboral tentacles with smaller capitula (45-70 μm), decreasing in size proximally. Living polyps transparent (Figure 5.8A). Medusa buds minute, originating from the hydrorhiza and grouped in clusters of up to four buds (Figure 5.8F). When released, medusae motionless and still for several hours. After about 10 hours, tentacles projected outside the bell cavity.

Medusa: Newly released medusae small, globular, up to 220 μm wide and 180 μm high (Figure 5.8G). Several macrobasic holotrichous mastigophores scattered over the thick exumbrella (Figure 5.8L). Manubrium relatively long (up to 150 μm) protruding from the bell cavity, with a terminal circular mouth with 4-5 lips (Figure 5.8J). Two opposite bulbs with small macrobasic holotrichous euryteles, initially inside the bell cavity and everted after two days (Figure 5.8H, I). Each bulb bearing a tenacle up to 600 μm, armed with 15-22 oval and hairy cnidophores (15 x 20 μm) borne on pedicels up to 70 μm long (Figure 5.8K). Each cnidophore containing 2-4 small macrobasic apotrichous euryteles (Figure 5.8M, N). living medusae transparent, with an orange gastric cavity (Figure 5.8I).

Cnidome: i) Small stenoteles (10 x 7 μm) in capitula. ii) Large stenoteles (24 x 19 μm) in capitula. iii) Large macrobasic holotrichous euryteles (20 x 14 μm; discharged capsule: 19 x 11 μm; shaft: 180 μm) in the hydrorhiza. iv) Macrobasic holotrichous mastigophores (10 x 10 μm; discharged capsule: 10 x 8 μm; shaft: 40 μm) scattered over the exumbrella. v) Small macrobasic apotrichous euryteles (7 x 4 μm; discharged capsule: 6 x 4 μm; shaft: 30 μm) in tentacular bulbs and in cnidophores.

Distribution: The new species is known from the reefs around Magoodhoo Island, Faafu Atoll, Republic of Maldives and from Thuwal and Al Lith, Saudi Arabia.

Remarks: *Zanclella labiata* is extremely similar to *Zanclella diabolica* in both the polyp and medusa stages. Both species have *Zanclea*-like polyps, hydrorhiza projecting out of the bryozoan skeleton, minute medusa buds borne on small pedicels from the hydrorhiza, newly released medusae small, globular and they share the same cnidome. However, slight differences are found in the protrusion of the hydrorhiza, since *Z. diabolica* shows typical clusters of nematocysts with also stenoteles, whereas in *Z. labiata* the protrusion are more irregular and contain only euryteles. Moreover, the polyps in *Z. labiata* are more slender, have more tentacles and do not have the typical white band at the base as in *Z. diabolica* (Figure 5.1J). Another difference is found in the medusa, since *Z. labiata* has a mouth with four lips, whereas *Z. diabolica* has no lips. The establishment of the new species is also supported by molecular analyses, being *Z. labiata* highly divergent from *Z. diabolica* and *Zanclella* sp. from Israel (Figure 5.3).

Etymology: The specific name refers to the typical lips found on the mouth of the medusa stage.

Zanclella sp.

Polyp: Colony stolonal, associated with the bryozoan *Rhynchozoon larreyi* (Audouin 1826). Polyps *Zanclea*-like.

Distribution: This species was found in the northern Red Sea (Eilat Bay, Israel).
Remarks: The colony was collected during the HyDRa Project (Pica et al. 2017) and identified as *Zanclea* sp. 1 associated with *R. larreyi*. No further information is available about the morphology but molecular analyses clearly clustered this species with the *Zanclella* clade. The bryozoan *R. larreyi* is known to host many *Zanclea* species, including *Zanclea retractilis* from Papua New Guinea (Boero et al. 2000), *Zanclea polymorpha* from New Zealand (Schuchert 1996), *Zanclea exposita* from Indonesia (Puce et al. 2002) and *Zanclea* sp. from Eilat and Port Sudan in the Red Sea (Ristedt and Schuhmacher 1985). The latter species may correspond to the here analysed *Zanclella* sp. since both are found in the same locality, but no conclusion can be drawn due to the lack of morphological data.

Zanclea sp. 1

Polyp: Colony stolonal, growing on the surface of shells of mussels (possibly *Mytilus* sp.). Hydrorhiza perisarc-covered, giving rise to monomorphic polyps. Reproduction occurs through a medusa stage.
Distribution: This species is found in Xiamen Bay, China.
Remarks: This species was collected from mussels attached to a concrete floating dock in Xiamen and then sequenced in four molecular markers within the project 'DNA Barcoding Medusozoa of China Sea'. No formal description is available at the moment, but one of the collectors provided some preliminary data about the morphology and ecology of the colonies. Specifically, this species is abundant in the investigated area and is highly similar to *Zanclea costata* in both its polyps and newly liberated medusae (Jinru He, personal communication).

Zanclea sp. 2

Medusa: Adult medusa up to 4.5 mm high, with two or four tentacles.
Distribution: The samples were collected in the Mediterranean Sea (Villefranche-sur-Mer, France) using plankton nets.
Remarks: This species is so far known only in its medusa stage and no identification at species level is available. The other *Zanclea* species known to develop up to four tentacles are *Zanclea costata* (Boero et al. 2000) and *Zanclea sessilis* (Schuchert 2010). However, *Zanclea* sp. 2 appears to be morphologically distinct from these two species (Peter Schuchert, personal communication). Also the molecular analyses confirmed that this species does not belong neither to *Z. costata* nor to *Z. sessilis* and may represent an undescribed species.

Zanclea sp. 3

Figure 5.9

Polyp: Colony stolonal, growing on a variety of substrates including bryozoans (Figure 5.9A) and crustose coralline algae (Figure 5.9B). Hydrorhiza perisarc-covered giving rise to monomorphic polyps through perisarc-covered and slightly annulated pedicels. Gastrozooid cylindrical, up to 1 mm high, with an apical mouth, five oral tentacles and 19-22 aboral tentacles scattered on the whole polyp (Figure 5.9C). Capitula of oral tentacles (70-80 μm) slightly bigger than those of aboral tentacles (55-70 μm), in all cases filled with stenoteles of two size classes (Figure 5.9D). Macrobasic apotrichous euryteles around the mouth and scattered in the polyp body wall (Figure 5.9E, F). Up to 10 medusa buds at different stages of maturation carried in the proximal half of the polyp. Living polyps transparent with white mouths (Figure 5.9A, B).

Medusa: Newly released medusa with a bell-shaped umbrella, with a diameter of 700-800 μm (Figure 5.9G). Manubrium reaching 2/5 of the subumbrellar cavity in length (250-270 μm), with a terminal, circular mouth surrounded by small stenoteles (Figure 5.9H). Four perradial canals ending in two big tentacular bulbs (Figure 5.9I) and two small bulbs without tentacles, linked by a circular canal. Large macrobasic apotrichous euryteles inside large bulbs and in the circular canal in correspondence of the small bulbs. Large bulbs with also small macrobasic apotrichous bean-shaped euryteles. Four perradial nematocyst pouches with large stenoteles starting close to the bulbs and running up to 1/3 of the length of the exumbrella (Figure 5.9J). Two tentacles armed with up to 45 rounded, hairy cnidophores (Figure 5.9K) containing 3-5 small macrobasic apotrichous bean-shaped euryteles (Figure 5.9L, M). Living medusae transparent with whitish mouths and bulbs (Figure 5.9G).

Cnidome: i) Small stenoteles (11x 9 μm) in capitula of polyps and mouth of medusae. ii) Large stenoteles (15 x 13 μm) in capitula of polyps and nematocyst pouches of medusae. iii) Large macrobasic apotrichous euryteles (22 x 10 μm; discharged capsule: 18 x 7 μm; shaft: 170 μm) around mouth and dispersed in body walls of polyps, and bulbs and circular canals of medusae. iv) Small macrobasic apotrichous bean-shaped euryteles (10 x 7 μm; discharged capsule: 8 x 5 μm; shaft: 35 μm) in bulbs and cnidophores.

Distribution: This species is so far known from reefs around Magoodhoo Island, Faafu Atoll, Republic of Maldives

Remarks: This morphology and size of gastrozooids and medusae of this species are identical to those of *Zanclea sango* and very similar to *Zanclea gilii* and *Zanclea fanella*. However, this species is not polymorphic and associated with scleractinians contrarily to *Z. sango* and *Z. gilii* (Boero et al. 2000, Hirose and Hirose 2011), and the newly released medusa of *Z. fanella* has isorhizas scattered on the exumbrella (Boero et al. 2000), whereas on the exumbrella of the species described above no nematocysts can be observed. Since only the newly liberated medusa was examined, further differences or similarities with other species may have been overlooked and due to the high resemblance with other species, this species is provisionally not identified at species level. Moreover, the phylogenetic analyses place this taxon very close to *Zanclea alba*, but these two organisms are morphologically distinct, especially regarding the cnidome and the shape of the newly liberated medusa (Calder 1988). Given these uncertainties, this organism may belong to an undescribed species.

5.5. DISCUSSION

The superfamily Zancleida is here confirmed to be a fully supported monophyletic group within the Capitata. The addition of previously unsampled taxa and new molecular markers allowed the reconstruction of a generally well supported phylogenetic hypothesis. The first diverging group is the clade composed of Pennariidae, Hydrocorynidae, Moerisiidae, and Halimedusidae. The Moerisiidae appears here to be paraphyletic and, to solve this issue, *Tiaricodon coeruleus* should be moved from the Halimedusidae to this family, but further investigations including other moerisiid and halimedusid species and genera should be performed before taking the taxonomic decision. The subsequent divergent group is the Sphaerocorynidae, followed by the Cladocorynidae and the Porpitidae. The remaining families, i.e. the Zancleidae, Asyncorynidae, Solanderiidae, and Milleporidae, form all together a fully supported monophyletic clade that shows nevertheless a confused within-group situation. Indeed, the family Zancleidae is highly polyphyletic, being subdivided in three not reciprocally monophyletic groups: Zancleidae 1, 2, and 3. The Zancleidae 1 is the sister group of all other taxa, the Zancleidae 2 is sister group of *Asyncoryne ryniensis*, even if the node is weakly supported, and the Zancleidae 3 is the sister group of the Milleporidae + Solanderiidae. According to this phylogenetic hypothesis, the Zancleidae 1 can be intended as the 'real' Zancleidae and *Zanclea*, since it includes the type species *Zanclea costata*. The Zancleidae 2 contains the medusa-based species *Zanclea prolifera* and may be merged with the Asyncorynidae, as already suggested by Nawrocki et al. (2010), also because *Zanclea* and *Asyncoryne* have very similar medusae. However, being *Z. prolifera* known only in its medusa stage (Uchida and Sugiura 1976) and being the nodal support of the relationship with *Asyncoryne ryniensis* low, no taxonomic decisions can be taken. The Zancleida 3 is likely to represent a new family of hydrozoans, being a divergent monophyletic group sister of the Milleporidae and Solanderiidae. The Zancleidae 1 contains so far the highest diversity of species, being composed of 15 *Zanclea* and one *Halocoryne* species. The type species *Z. costata* is the sister species of all other taxa and is considered a Mediterranean endemic (Schuchert 2010). The other temperate species (*Zanclea sessilis*, *Zanclea implexa*, *Zanclea giancarloi*, and *Zanclea* sp. 2) are found in the Mediterranean and the North-Eastern Atlantic and form a monophyletic clade. Within this group, *Z. sessilis* and *Z. implexa* are very similar species and were previously considered as conspecific (Russell and Rees 1936). However, Schuchert (2010) treated them as two different species, mainly due to the association of *Z. sessilis* with bryozoans and the constant presence of a perisarc-covered pedicel in *Z. implexa*. This idea is here confirmed with molecular phylogenetics data and the moderately high genetic distance between the two taxa (*16s rRNA*: 3.7 ± 0.6 %). *Zanclea* sp. 2 could not be identified at species level and may correspond to an undescribed species. Indeed, according to the few morphological data, it is similar to *Z. sessilis* and *Z. costata* but the molecular results do not place it close to none of these two species. The other species clustering in the Zancleidae 1 are *Zanclea* sp. 1 from China, for which few morphological data are available, the coral-associated *Zanclea* group and *Halocoryne pirainoid*. Unexpectedly, the latter species clusters with coral-associated-*Zanclea*, far from the other *Halocoryne* species included in the analysis. *Halocoryne pirainoid* is similar to the polyp of *Halocoryne epizoica*, mostly differing

in the cnidome and the colouration of polyps (likely due to different pigmentation of the bryozoan hosts) and this morphological similarity seems therefore to be due to a convergent reduction of the polyp stage. However, *H. pirainoid* produces a typical *Zanclea* medusa, whereas *H. epizoica* produces a reduced eumedusoid and this feature may partially explain their great divergence, even if in other hydrozoan taxa the occurrence of medusa or eumedusoid is not linked to high genetic divergence and do not justify separation of distinct genera (e.g. Miglietta et al. 2009). The Zancleidae 3 are composed of three tropical *Zanclea* species, the Mediterranean *H. epizoica*, and the tropical genus *Zanclella*. These taxa cluster in two main clades. A first clade includes the euryteles-lacking species *Zanclea eilatensis*, *Zanclea protecta*, and *H. epizoica*. *Zanclea eilatensis* and *Z. protecta* are highly similar in both the polyp and medusa stages and their close relationships is also confirmed by molecular analyses. Contrarily, both polyp and medusa stages of *H. epizoica* are highly different from those of the two *Zanclea* species and this is the only temperate species in the group. The second clade of the Zancleidae 3 contains *Zanclea divergens* and three species of *Zanclella.* All the *Zanclella* species included in the analysis have a *Zanclea*-like polyp and are monophyletic. The medusae of *Zanclella diabolica* and *Zanclella labiata* are very similar with each other and quite different from *Zanclea* medusae. Other described *Zanclella* species have reduced polymorphic polyps and the type species *Zanclella bryozoophila* also reproduces through a reduced eumedusoid stage (Boero and Hewitt 1992, Boero et al. 2000). Therefore, a morphological diagnosis of the genus *Zanclella* remains hard to be defined and only further molecular analyses could clarify if the *Zanclella* species with reduced polyps actually belong to the same group of the herein included *Zanclea*-like *Zanclella* species. *Zanclea divergens* was previously known only from the Pacific Ocean, specifically from Papua New Guinea (type locality) and Indonesia (Boero et al. 2000, Puce et al. 2002) and the distribution is here widened to the Indian Ocean and the Red Sea. All the specimens collected were identifiable as *Z. divergens* thanks to both polyp and medusa features but molecular analyses showed a certain level of genetic structuring within this morphospecies. Two of the four main molecular lineages are found sympatrically in the Maldives, one in the Saudi Arabian Red Sea, and another one, possibly the type species, in Indonesia. These clades may correspond to different cryptic species, due to their great genetic divergence also confirmed by *16S rRNA* genetic distances. However, species delimitation techniques were not used to solve this issue due to the undersampling of some of the lineages.

The ancestral states reconstructions show that the investigated characters exhibit high plasticity and recurrent events of independent evolution leading to similar morphologies or preferences in not strictly related clades of the Zancleida. Previous studies focusing on other taxa (Miglietta and Cunningham 2012, Puce et al. 2016) or on the whole class Hydrozoa (Cartwright and Nawrocki 2010) depicted a similar scenario, with many cases of reversal, parallelism and convergence. The symbiotic lifestyle arose multiple times in the Zancleida, at least four, and, even if in most associations the nature of the relationships has not been elucidated yet, in some cases a highly specialised interaction has been documented (e.g. the parasitism of *H. epizoica* on the bryozoan *Schizobrachiella sanguinea* (Norman 1868) described by Piraino et al. (1992)). Some species are known to live both in association with multiple organisms and also on abiotic substrates, suggesting a facultative association with their host such as in the case of *Z. giancarloi*, *Z. implexa* and *Zanclea* sp.3 (Gravili et al. 1996,

Schuchert 2010). All other symbiotic species are specifically associated with a single host or with a group of closely related organisms. The association with scleractinians, octocorals, and sponges emerged one time in the most recent common ancestors of coral-associated *Zanclea*, octocorals-associated Cladocorynidae, and Sphaerocorynidae, respectively, and led in some cases to very host-specific relationships. Conversely, the association with bryozoans appeared different times in divergent lineages. It is not clear whether the relationship with bryozoans evolved one or more times within the Zancleidae 1, whereas, according to the analysis, this feature was likely to be already present in the ancestor of the Zancleidae 3, since all representatives of this clade are specifically associated with bryozoans. Previous authors hypothesised some evolutionary trends in symbiotic species, including a tendency towards polymorphism, specialisation of the hydrorhiza (e.g. loss of perisarc and protection due to the host skeleton or tissues), proximal and stolonal medusa buds, and a reduction of the complexity of both polyp and medusa stages (Boero et al. 2000, Puce et al. 2002, Puce et al. 2008, Miglietta and Cunningham 2012). Cartwright and Nawrocki (2010) showed that polymorphism is found in all major Hydroidolina clades (except Aplanulata), suggesting that the division of labour is an important evolutionary innovation in colonial hydrozoans. Polymorphism was gained several times in the Zancleida but, apparently, not in a manner dependent from the symbiotic lifestyle, and was also lost in the ancestor of the specifically-associated *Zanclea intermedia* II and III. Polyp specialisation occurred convergently in the ancestors of the calcifying Milleporidae, the pelagic Porpitidae, in *Z. sessilis* and probably in the ancestor of *H. pirainoid* and coral-associated *Zanclea.* All other symbiotic and non-symbiotic species are so far considered monomorphic, even if dactylozooids can be easily overlooked and in some cases are facultative (Altuna 2016), leaving open the possibility that they could be present also in other species. According to these results, a subdivision of *Zanclea* in polymorphic and monomorphic colonies (Boero et al. 2000) is therefore not supported by phylogenetic results. The perisarc loss in symbiotic Zancleidae is thought to be linked to an increased protection due to the overgrowth of the host skeleton over the hydrorhiza (Puce et al. 2002). Indeed, the hydrorhiza is naked only in symbiotic taxa and only when overgrown by host skeleton or tissues, with the only exception of *Zanclea exposita*. The loss of perisarc seems to have occurred four times in the Zancleida, specifically in *H. pirainoid*, in *Zanclea gallii* I and II, in the common ancestor of the Zancleida 3 and in *Pseudozanclea timida*. However, most of the symbiotic species with the hydrorhiza completely embedded in host tissues, and in some cases also overgrown by host skeleton, still have the perisarc covering the hydrorhiza, and this could be related to a possible not strictly positive association with their host. For instance, alcyoniids and sponges are known to produce a wide range of secondary metabolites (Pawlik 1993) that could have negative effects also on the hydroids and therefore could disadvantage the selection of a perisarc-free hydrorhiza. In the Zancleidae 1, a certain trend can be observed, even if with some exceptions, with the earliest diverging species living on the surface of their host and the more derived species having the hydrorhiza surrounded by the host skeleton and in some cases without perisarc. Interestingly, the coral hosts of the perisarc-free *Z. gallii* I and II do not overgrow the hydrorhiza with their skeleton, contrarily to what happens in other coral-associated species with perisarc. The presence of a skeletal layer surrounding the hydrorhiza could possibly favour the loss of perisarc, but the absence of this skeletal structures could reflect

an even more integrated association, with the host and symbiont tissues in close contact. However, further studies are needed to investigate the possible exchanges and interactions at the interface between the host and symbiont in these intimate relationships. Puce et al. (2002) linked the position of medusa buds in symbiotic Zancleidae to the level of specialisation of the hydrorhiza, suggesting that species intimately associated with their host have a naked hydrorhiza and medusa buds borne on pedicels arising directly from the hydrorhiza. According to the analysis here presented, the situation seems to be more complex and no clear evolutionary trends can be observed. However, in most species deprived of perisarc the buds are stolonal, with the exception of *Halocoryne* species and *P. timida.* Interestingly, the Sphaerocorynidae are the only symbiotic species with buds carried on the oral half of the polyp and this may support again the fact that the sponge-hydrozoan interaction might not be completely positive for the hydroid symbiont. The reduction of the medusa stage occurred several times in the evolution of Hydroidolina (Cartwright and Nawrocki 2010) and these events are correlated to the evolution of the colony shape in the Leptothecata (Leclère et al. 2009). In the Zancleida there is no association between medusa reduction and an increase in host specificity (Boero 1984) but upright colonies (including calcifying milleporids) have their medusa stage reduced to some extent, probably due to similar ecological pressures as in the Leptothecata (Leclère et al. 2009). The reduction of the polyp stage is mostly found in the genera *Halocoryne* and *Zanclella* (Boero et al. 2000). The two *Halocoryne* species included in the analyses, i.e. *H. epizoica* and *H. pirainoid*, show similar reduced polyp stages but are clearly divergent and this trait seems to be a convergence. These two species have similar feeding haehaviour, since they both feed on lophophoral tenacles of the host (Piraino et al. 1992, Boero et al. 2000, personal observation) and this habit may have driven the convergent reduction of the polyp stage. However, closer examinations of the inter-specific interactions between hydrozoans and their hosts and their adaptive outcomes in the benthic community are needed to better address this issue.

Overall, the investigated characters show a complex evolution, with several gains, losses and re-gains, making difficult to detect a clear and general evolutionary trend for symbiotic species. However, a moderate level of convergence in highly integrated symbiotic species can be observed. The plasticity of these characters, along with the complicated systematics of the group highlighted by the phylogenetic analyses, do not even allow to establish clear morphological diagnoses for the recovered clades and to take undoubted taxonomic decisions. Additionally, the presence of cryptic species has been revealed in coral-associated *Zanclea* and *Z. divergens* and it is likely that other morphospecies are hiding species complexes. Russell and Rees (1936) and other following authors recognised only a few variable species of *Zanclea* worldwide, but this has been demonstrated to be incorrect and the genus *Zanclea* is now considered as a speciose group, with new species described more or less regularly. This idea is confirmed by the present work, which demonstrates the validity of species previously considered conspecific, the presence of cryptic species, and the presence of still unknown species of both *Zanclea* and *Zanclella*. However, the diversity of the family Zancleidae is still likely to be underestimated, since many species have the life cycle not completely known and are still to be included in molecular analyses. Moreovoer, the knowledge of the ecological preferences and interactions with their host could possibly shed more light on the evolution and the ecological significance of these species. For instance,

some Zancleidae species are thought to be involved in mutualistic association with their hosts, favouring not only themselves but also their bryozoans and corals hosts (e.g. Osman and Haugsness (1981), Ristedt and Schuhmacher (1985), Montano et al. (2017)). In other cases, the situation is not completely clear, such as for *H. epizoica*, which can be considered as a parasite of the bryozoan host and at the same time it could have a defensive role against predators and enhance the competition success of both itself and the host (Piraino et al. 1992). The understanding of how these ecological interactions may mediate the response of species to biotic and abiotic disturbances is of undoubted importance, especially in relation to the effects of the ongoing climate changes.

5.6. REFERENCES

Agassiz L (1862) *Contributions to the Natural History of the United States of America: Second Monograph*. Little, Brown and Company.

Altuna Á (2016) The life cycle of symbiotic *Zanclea sessilis* (Hydrozoa: Zancleidae) colonies with polymorphic hydranths from the Bay of Biscay. *Marine Biodiversity* 46: 901-910.

Boero F (1984) The ecology of marine hydroids and effects of environmental factors: a review. *Marine Ecology* 5: 93-118.

Boero F, Bouillon J, Gravili C (2000) A survey of *Zanclea*, *Halocoryne* and *Zanclella* (Cnidaria, Hydrozoa, Anthomedusae, Zancleidae) with description of new species. *Italian Journal of Zoology* 67: 93-124.

Boero F, Hewitt C (1992) A hydrozoan, *Zanclella bryozoophila* n. gen., n. sp.(Zancleidae), symbiotic with a bryozoan, with a discussion of the Zancleoidea. *Canadian Journal of Zoology* 70: 1645-1651.

Bouillon J, Gravili C, Gili J-M, Boero F (2006) *An Introduction to Hydrozoa.* Publications Scientifiques du Museum, Paris.

Bouillon J, Pagès F, Gili J-M, Palanques A, Puig P, Heussner S (2000) Deep-water Hydromedusae from the Lacaze-Duthiers submarine canyon (Banyuls, northwestern Mediterranean) and description of two new genera, *Guillea* and *Parateclaia*. *Scientia Marina* 64: 87-95.

Browne ET (1916) IV. Medusæ from the India Ocean (Collected by Prof. Stanley Gardiner, in HMS "Sealark" in 1905). *Transactions of the Linnean Society of London* 17: 169-210.

Calder DR (1988) Shallow-Water Hydroids of Bermuda: The Athecatae. *Royal Ontario Museum Life Sciences Contributions* 148: 1-107.

Cartwright P, Nawrocki AM (2010) Character evolution in Hydrozoa (phylum Cnidaria). *Integrative and Comparative Biology* 50: 456-472.

Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution* 17: 540-552.

Collins AG, Winkelmann S, Hadrys H, Schierwater B (2005) Phylogeny of Capitata and Corynidae (Cnidaria, Hydrozoa) in light of mitochondrial 16S rDNA data. *Zoologica Scripta* 34: 91-99.

Cunningham CW, Buss LW (1993) Molecular evidence for multiple episodes of paedomorphosis in the family Hydractiniidae. *Biochemical Systematics and Ecology* 21: 57-69.

Drummond A, Ashton B, Buxton S, Cheung M, Cooper A, Heled J et al., Sturrock S (2010) Geneious v6. 1.6. *Website: http://www. geneious. com*.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* 3: 294-299.

Fontana S, Keshavmurthy S, Hsieh HJ, Denis V, Kuo C-Y, Hsu C-M et al., Chen CA (2012) Molecular evidence shows low species diversity of coral-associated hydroids in *Acropora* corals. *PloS One* 7: e50130.

Galea HR (2008) On a collection of shallow-water hydroids (Cnidaria: Hydrozoa) from Guadeloupe and Les Saintes, French Lesser Antilles. *Zootaxa* 1878: 1-54.

Gershwin LA, Zeidler W (2003) Encounter 2002 expedition to the isles of St Francis, South Australia: medusae, siphonophores and ctenophores. *Transactions of the Royal Society of South Australia* 127: 205-241.

Gravili C, Boero F, Bouillon J (1996) *Zanclea* species (Hydroidomedusae, Anthomedusae) from the Mediterranean. *Scientia Marina* 60: 99-108.

Haeckel EHPA (1879) *Monographie der Medusen*. G. Fischer.

Hirose M, Hirose E (2011) A new species of *Zanclea* (Cnidaria: Hydrozoa) associated with scleractinian corals from Okinawa, Japan. *Journal of the Marine Biological Association of the United Kingdom* 92: 877-884.

Jankowski T (2001) The freshwater medusae of the world-a taxonomic and systematic literature study with some remarks on other inland water jellyfish. *Hydrobiologia* 462: 91-113.

Katoh K, Standley DM (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution* 30: 772-780.

Kayal E, Bentlage B, Cartwright P, Yanagihara AA, Lindsay DJ, Hopcroft RR, Collins AG (2015) Phylogenetic analysis of higher-level relationships within Hydroidolina (Cnidaria: Hydrozoa) using mitochondrial genome data and insight into their mitochondrial transcription. *PeerJ* 3: e1403.

Kramp P (1959) Some new and little-known Indo-Pacific medusae. *Videnskabelige Meddelelser fra Dansk Naturhistorisk Forening* 121: 223-259.

Kramp P (1968) The Hydromedusae of the Pacific and Indian Oceans. Sections II and III. *Dana Report* 72: 1-200.

Lanfear R, Calcott B, Ho SY, Guindon S (2012) PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution* 29: 1695-1701.

Leclère L, Schuchert P, Cruaud C, Couloux A, Manuel M (2009) Molecular phylogenetics of Thecata (Hydrozoa, Cnidaria) reveals long-term maintenance of life history traits despite high frequency of recent character changes. *Systematic Biology* 58: 509-526.

Lewis PO (2001) A likelihood approach to estimating phylogeny from discrete morphological character data. *Systematic Biology* 50: 913-925.

Maddison WP, Maddison DR (2001) Mesquite: a modular system for evolutionary analysis.

Maggioni D, Montano S, Seveso D, Galli P (2016) Molecular evidence for cryptic species in *Pteroclava krempfi* (Hydrozoa, Cladocorynidae) living in association with alcyonaceans. *Systematics and Biodiversity* 14: 484-493.

Mangin KL (1991) Community effects of the invasion of a new intertidal hydroid, *Samuraia tabularasa*, in the Gulf of California. PhD thesis, University of Arizona.

Medlin L, Elwood HJ, Stickel S, Sogin ML (1988) The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene* 71: 491-499.

Miglietta MP, Schuchert P, Cunningham CW (2009) Reconciling genealogical and morphological species in a worldwide study of the Family Hydractiniidae (Cnidaria, Hydrozoa). *Zoologica Scripta* 38: 403-430.

Miglietta MP, Cunningham CW (2012) Evolution of life cycle, colony morphology, and host specificity in the family Hydractiniidae (Hydrozoa, Cnidaria). *Evolution* 66: 3876-3901.

Miglietta MP, Odegard D, Faure B, Faucci A (2015) Barcoding Techniques Help Tracking the Evolutionary History of the Introduced Species *Pennaria disticha* (Hydrozoa, Cnidaria). *PloS One* 10: e0144762.

Montano S, Fattorini S, Parravicini V, Berumen ML, Galli P, Maggioni D, Arrigoni R, Seveso D, Strona G. (2017). Corals hosting symbiotic hydrozoans are less susceptible to predation and disease. *Proceedings of the Royal Society B: Biological Sciences* 284: 20172405.

Nawrocki AM, Schuchert P, Cartwright P (2010) Phylogenetics and evolution of Capitata (Cnidaria: Hydrozoa), and the systematics of Corynidae. *Zoologica Scripta* 39: 290-304.

Osman RW, Haugsness JA (1981) Mutualism among sessile invertebrates: a mediator of competition and predation. *Science* 211: 846-848.

Pantos O, Bythell JC (2010) A novel reef coral symbiosis. *Coral Reefs* 29: 761-770.

Pawlik JR (1993) Marine invertebrate chemical defenses. *Chemical Reviews* 93: 1911-1922.

Peña-Cantero ÁL, Sentandreu V (2017) Phylogenetic relationships of endemic Antarctic species of *Staurotheca* Allman, 1888 (Cnidaria, Hydrozoa). *Polar Biology* 40: 301-312.

Peña Cantero ÁL, Boero F, Piraino S (2013) Shallow-water benthic hydroids from Tethys Bay (Terra Nova Bay, Ross Sea, Antarctica). *Polar Biology* 36: 731-753.

Petersen KW (1990) Evolution and taxonomy in capitate hydroids and medusae (Cnidaria: Hydrozoa). *Zoological Journal of the Linnean Society* 100: 101-231.

Pica D, Bastari A, Vaga CF, Di Camillo CG, Montano S, Puce S (2017) Hydroid diversity of Eilat Bay with the description of a new *Zanclea* species. *Marine Biology Research* 13: 469-479.

Piraino S, Bouillon J, Boero F (1992) *Halocoryne epizoica* (Cnidaria, Hydrozoa), a hydroid that "bites". *Scientia Marina* 56: 141-147.

Puce S, Bavestrello G, Di Camillo CG, Boero F (2007) Symbiotic relationships between hydroids and bryozoans. *Symbiosis* 44: 137-143.

Puce S, Cerrano C, Boyer M, Ferretti C, Bavestrello G (2002) *Zanclea* (Cnidaria: Hydrozoa) species from Bunaken Marine Park (Sulawesi Sea, Indonesia). *Journal of the Marine Biological Association of the United Kingdom* 82: 943-954.

Puce S, Cerrano C, Di Camillo CG, Bavestrello G (2008) Hydroidomedusae (Cnidaria: Hydrozoa) symbiotic radiation. *Journal of the Marine Biological Association of the United Kingdom* 88: 1715-1721.

Puce S, Pica D, Schiaparelli S, Negrisolo E (2016) Integration of Morphological Data into Molecular Phylogenetic Analysis: Toward the Identikit of the Stylasterid Ancestor. *PloS One* 11: e0161423.

Ristedt H, Schuhmacher H (1985) The bryozoan *Rhynchozoon larreyi* (Audouin, 1826)-A successful competitor in coral reef communities of the Red Sea. *Marine Ecology* 6: 167-179.

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S et al., Huelsenbeck JP (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* 61: 539-542.

Russell F, Rees W (1936) On rearing the hydroid *Zanclea implexa* (Alder) and its medusa *Zanclea gemmosa* McCrady, with a review of the genus Zanclea. *Journal of the Marine Biological Association of the United Kingdom* 21: 107-130.

Schuchert P (1996) *The Marine Fauna of New Zealand: Athecate Hydroids and Their Medusae*. New Zealand Oceanographic Institute.

Schuchert P (2001) Survey of the family Corynidae (Cnidaria, Hydrozoa). *Revue Suisse de Zoologie* 108: 739-878.

Schuchert P (2010) The European athecate hydroids and their medusae (Hydrozoa, Cnidaria): Capitata part 2. *Revue Suisse de Zoologie* 117: 337-555.

Stepanjants S (1972) Hydroidea of the coastal waters of the Davis Sea (collected by the XIth Soviet Antarctic Expedition of 1965-1966). *Biological Reports of the Sovietic Antarctic Expedition* 5: 56-79.

Sukumaran J, Holder MT (2010) DendroPy: a Python library for phylogenetic computing. *Bioinformatics* 26: 1569-1571.

Talavera G, Castresana J (2007) Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Systematic Biology* 56: 564-577.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution* 30: 2725-2729.

Uchida T, Sugiura Y (1976) On a Hydromedusa, *Zanclea prolifera* n. sp., of which the medusa gives rise to medusa-buds. *Proceedings of the Japan Academy* 52: 141-144.

Varela C (2012) Registros nuevos de hidrozoos (Cnidaria: Hydroidomedusae) para Cuba, con la descripción de una especie nueva. *Solenodon* 10: 1-7.

Xu Z, Huang J, Chen X (1991) On new species and record of Hydromedusae in the upwelling region off the Minnan-Taiwan Bank fishing ground, China. *Minnan-Taiwan Bank Fishing Ground Upwelling Ecosystem Study*. Beijing Science Press: 469: 486.

Xu Z, Huang J, Guo D (2008). Six new species of Anthomedusae (Hydrozoa, Hydroidomedusae) from Beibu Gulf, China. *Symposium on Oceanography of the Beibu Gulf*. China Ocean Press..

Zietara MS, Arndt A, Geets A, Hellemans B, Volckaert FA (2000) The nuclear rDNA region of *Gyrodactylus arcuatus* and *G. branchicus* (Monogenea: Gyrodactylidae). *Journal of Parasitology* 86: 1368-1373.

Zwickl D (2006) GARLI: genetic algorithm for rapid likelihood inference. *See http://www. http://www.bio.utexas.edu/faculty/antisense/garli/Garli.html*.

5.7. TABLES

Table 5.1. Genetic distances (*16S rRNA*, uncorrected p-distances in %) for the *Zanclea divergens* species complex. Each number (1-4) corresponds to a clade recovered in the phylogenetic tree (Figure 5.3). Values are indicated as mean ± standard deviation. n.c. not calculated.

16S rRNA	1)	2)	3)	4)
1) Indonesia	n.c.			
2) Maldives	8.4 ± 1.3	n.c.		
3) Saudi Arabia	9.7 ± 1.2	8.9 ± 1.3	0.3 ± 0.2	
4) Maldives	10.5 ± 1.4	9.4 ± 1.4	9.4 ± 1.4	1.2 ± 0.3

5.8. FIGURES

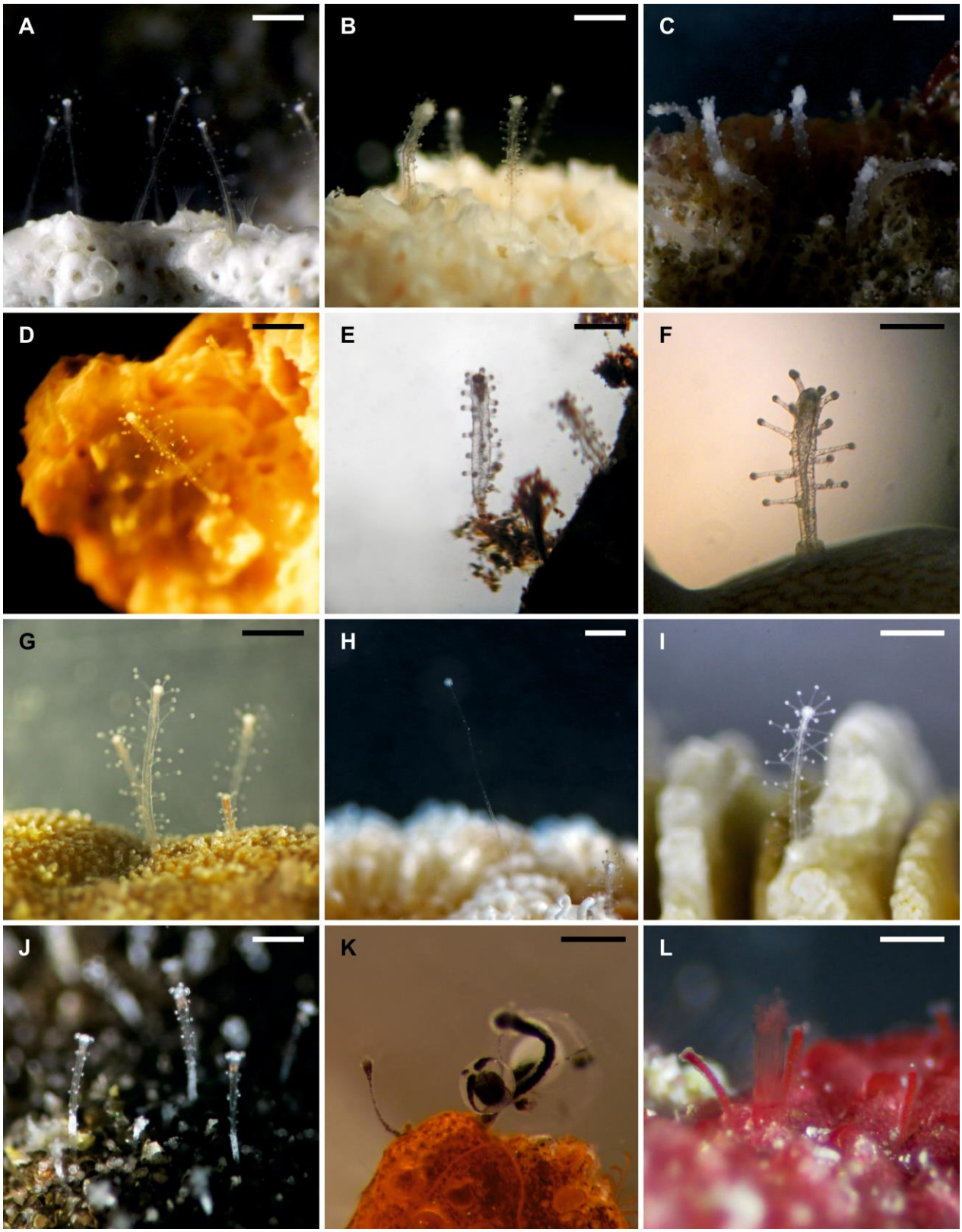

Figure 5.1. Zancleidae polyps. A) *Zanclea protecta*, B) *Zanclea eilatensis*, C) *Zanclea divergens*, D) *Zanclea sessilis*, E) *Zanclea alba*, F) *Zanclea gallii* II, G) *Zanclea intermedia* III, H) *Zanclea intermedia* I (dactylozooid), I) *Zanclea sango* II, J) *Zanclella diabolica*, K) *Halocoryne epizoica*, L) *Halocoryne pirainoid*. Scale bars: ~ 0.5 mm.

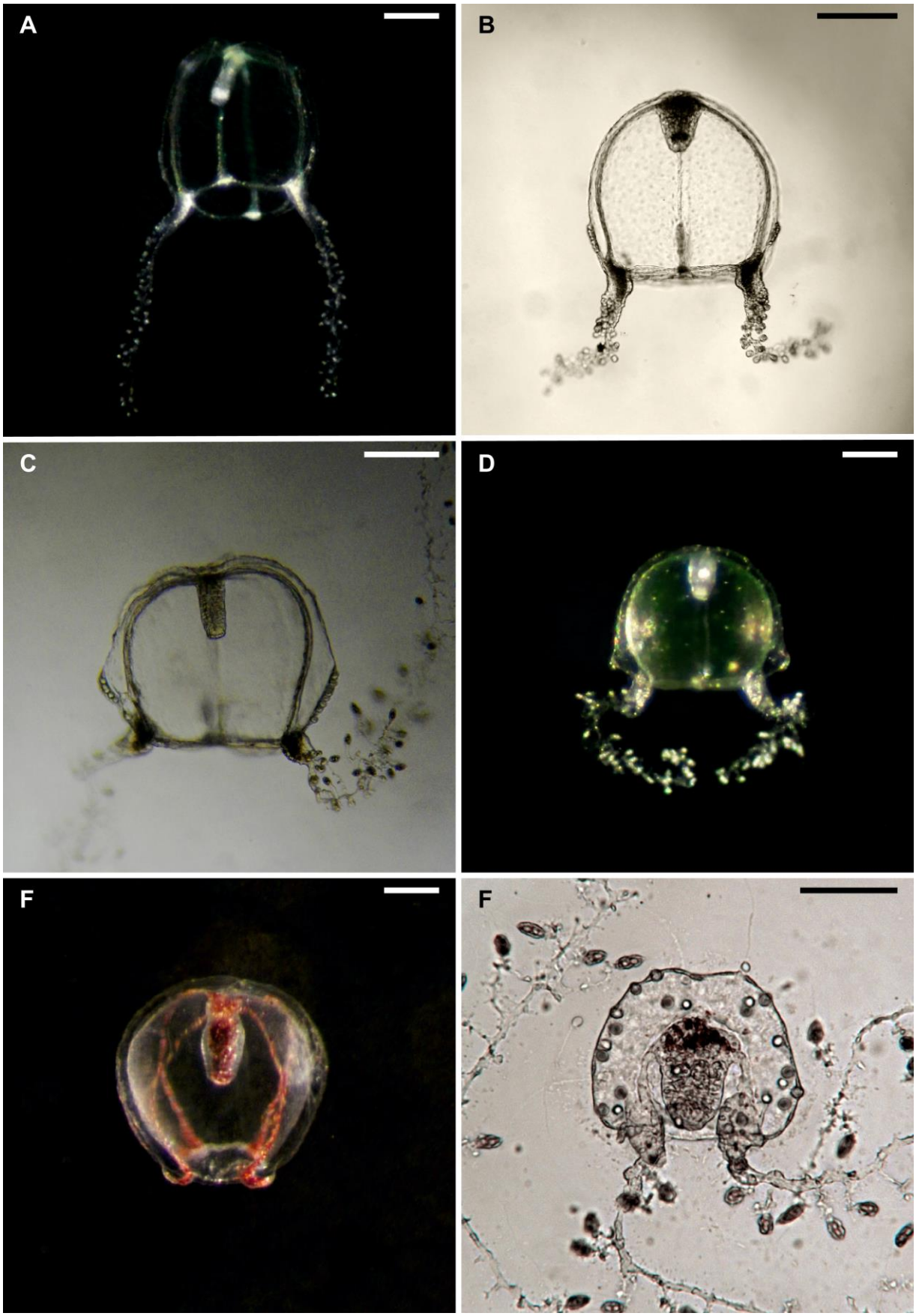

Figure 5.2. Zancleidae medusae. A) *Zanclea sango* II, B) *Zanclea intermedia* I, C) *Zanclea divergens*, D) *Zanclea protecta*, E) *Halocoryne epizoica* (eumedusoid), F) *Zanclella diabolica*. Scale bars: ~ 250 μm.

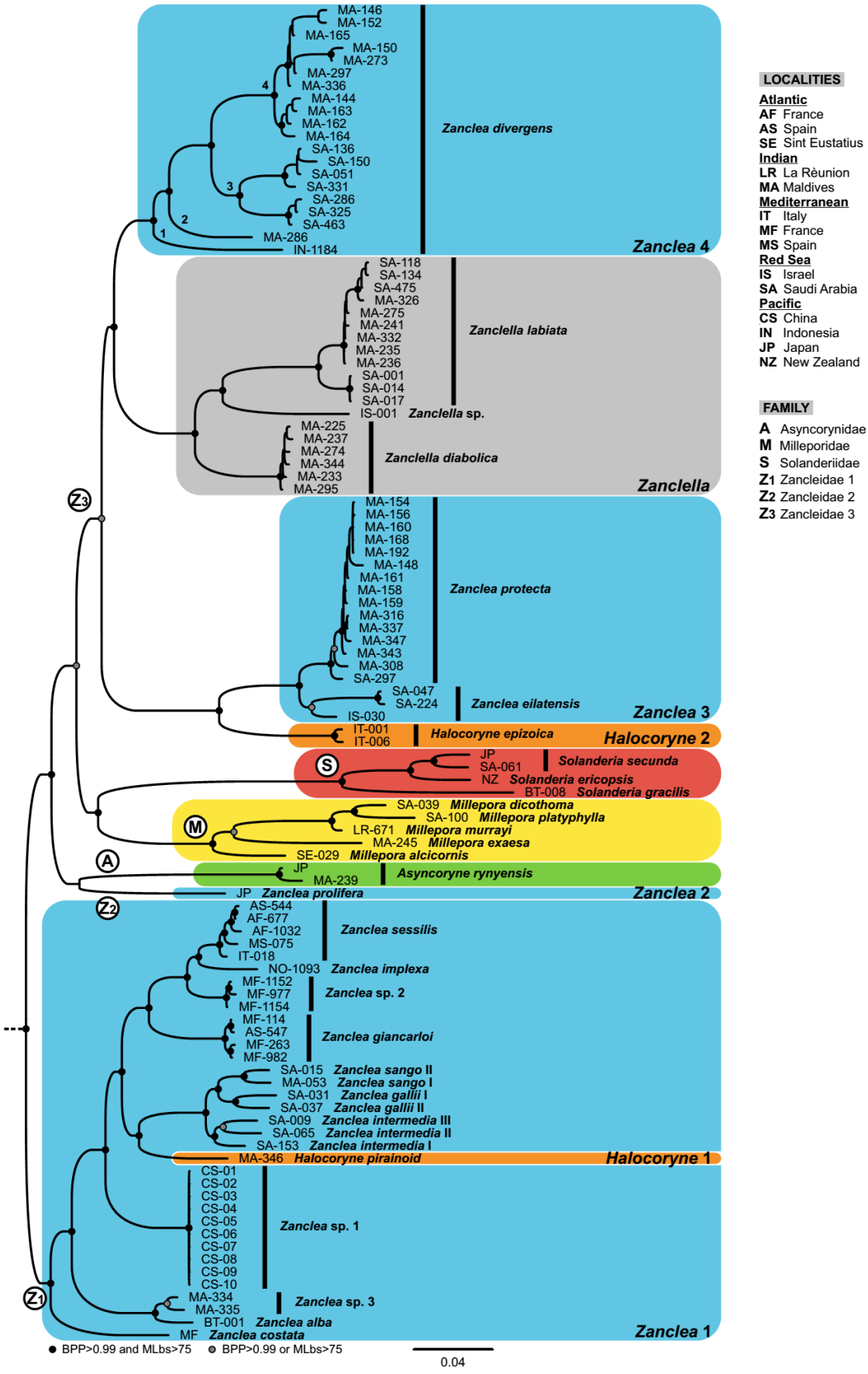

Figure 5.3. Bayesian phylogenetic hypothesis for the Zancleidae based on the concatenated mitochondrial and nuclear genes. Each genus is highlighted with a different colour and each family is indicated as a circle with letters as coded in the legend. For *Zanclea divergens*, each main clade is numbered (1-4).

Figure 5.4. Cladogram of the evolutionary relationships within the superfamily Zancleida (branch length not shown). Nodal supports are indicated as BPP and BS and each family is highlighted with a different colour.

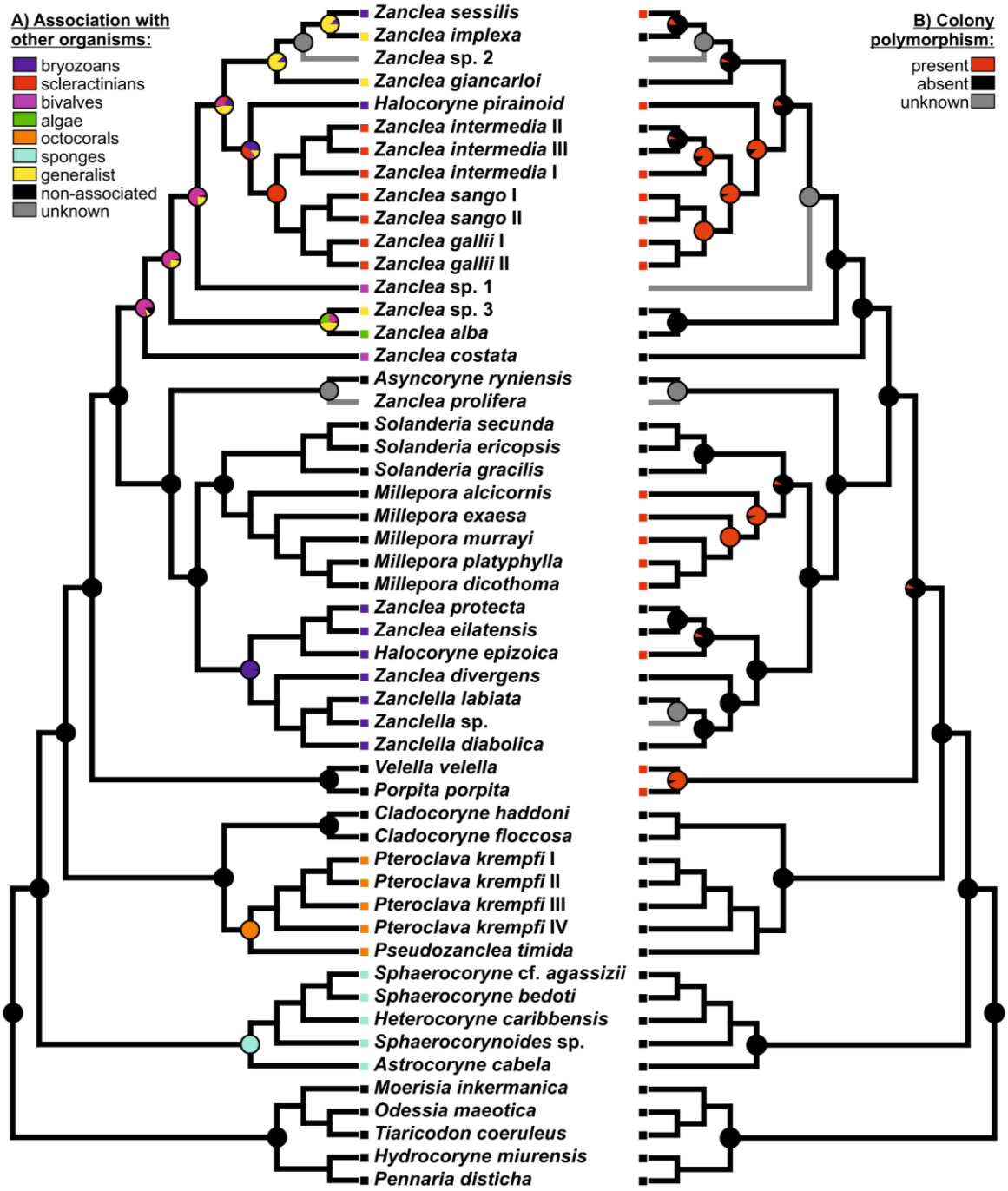

Figure 5.5. Evolution of the characters A) association with other organisms and B) polymorphism of the colony in the Zancleida. The state of the analysed character is represented by a square, placed at each terminal node, and by a pie at the internal nodes of the tree. When multiple states of a character occur at a specific node, the size of each slice is proportional to the probability of occurrence of the state. When the probability for a state is maximal for all nodes within a monophyletic group, the pie is shown only in the most recent common ancestor of the group.

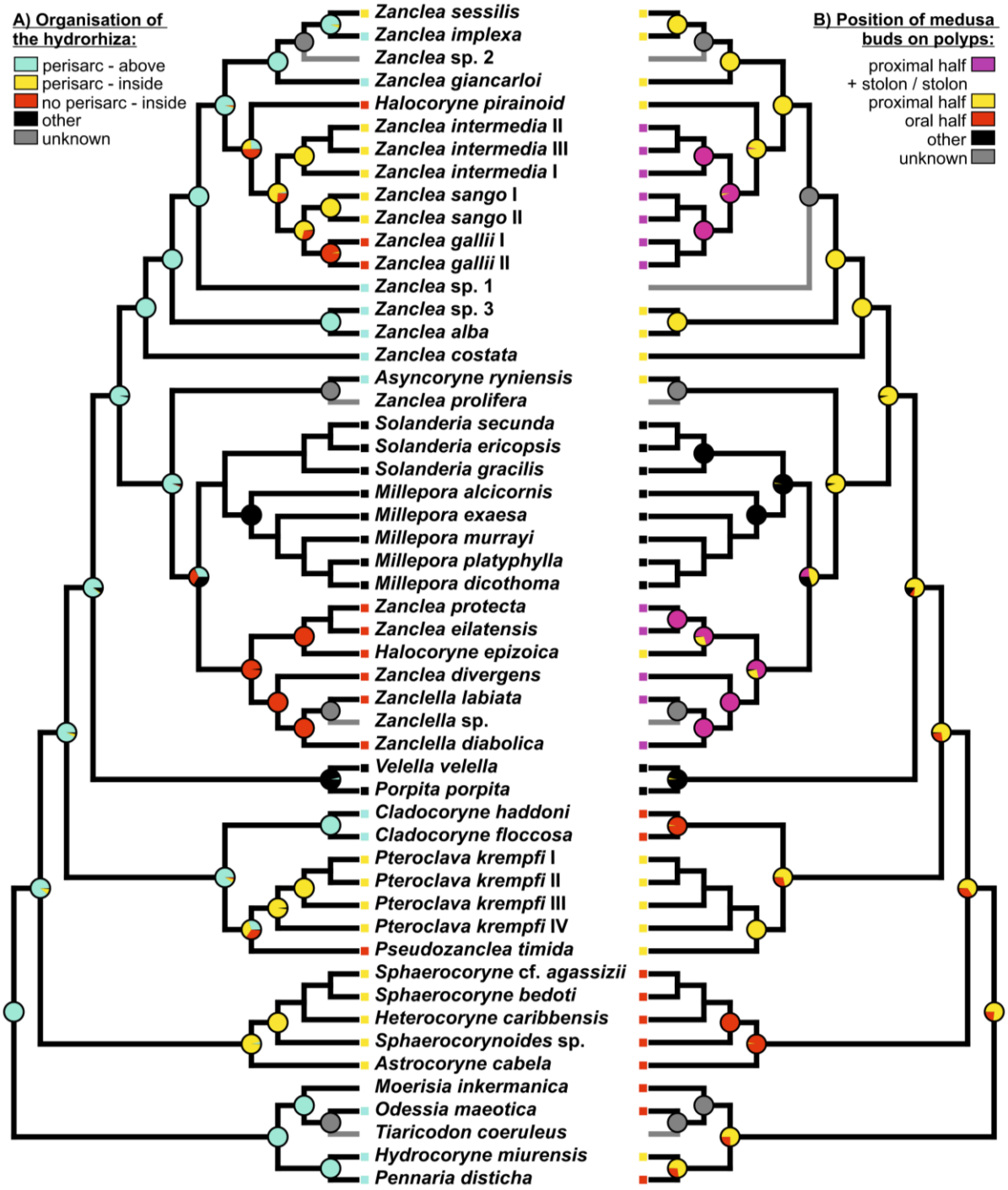

Figure 5.6. Evolution of the characters A) organisation of the hydrorhiza and B) position of the medusa buds in the Zancleida. The state of the analysed character is represented by a square, placed at each terminal node, and by a pie at the internal nodes of the tree. When multiple states of a character occur at a specific node, the size of each slice is proportional to the probability of occurrence of the state. When the probability for a state is maximal for all nodes within a monophyletic group, the pie is shown only in the most recent common ancestor of the group.

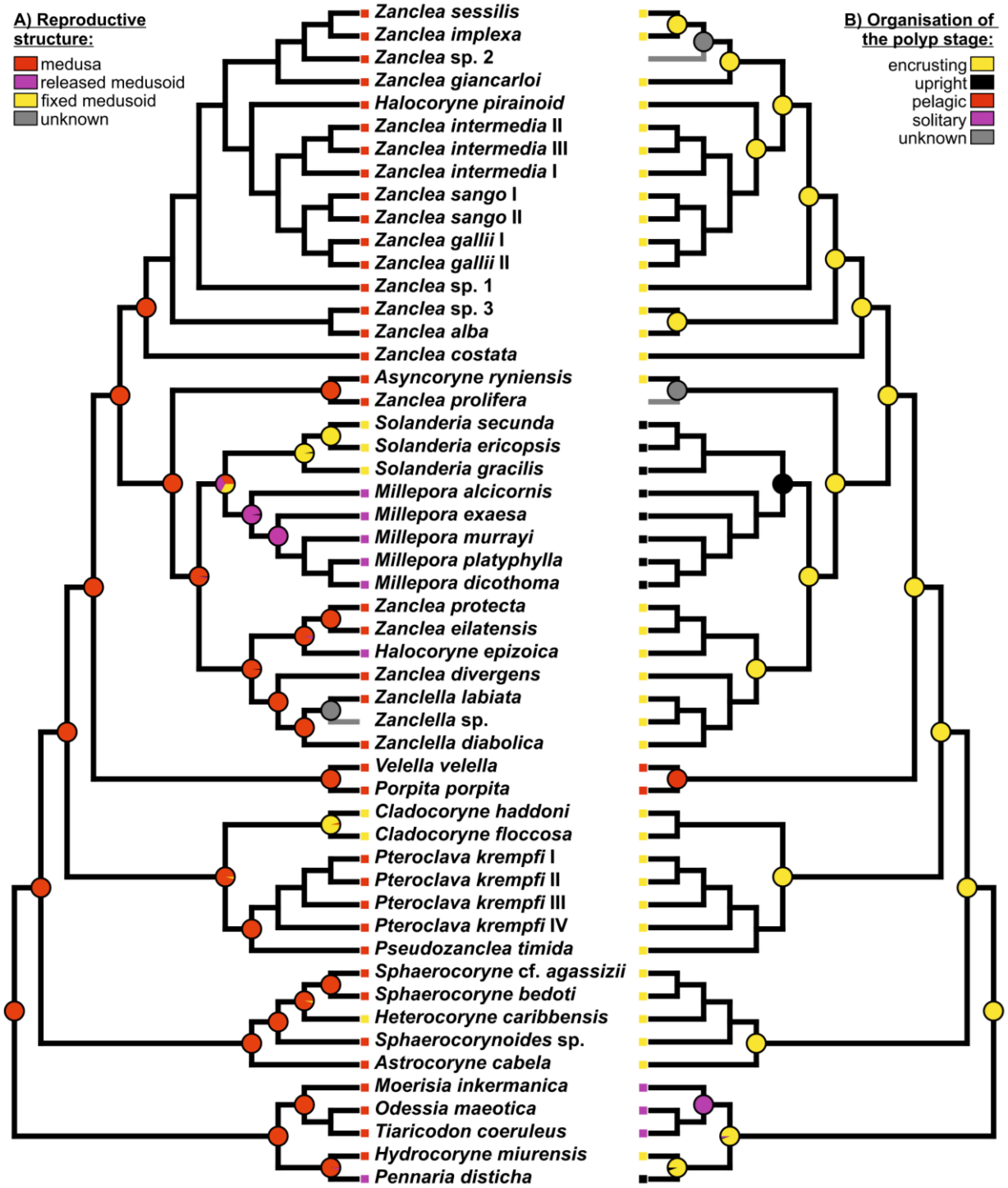

Figure 5.7. Evolution of the characters A) reproductive structure and B) organisation of the polyp stage in the Zancleida. The state of the analysed character is represented by a square, placed at each terminal node, and by a pie at the internal nodes of the tree. When multiple states of a character occur at a specific node, the size of each slice is proportional to the probability of occurrence of the state. When the probability for a state is maximal for all nodes within a monophyletic group, the pie is shown only in the most recent common ancestor of the group.

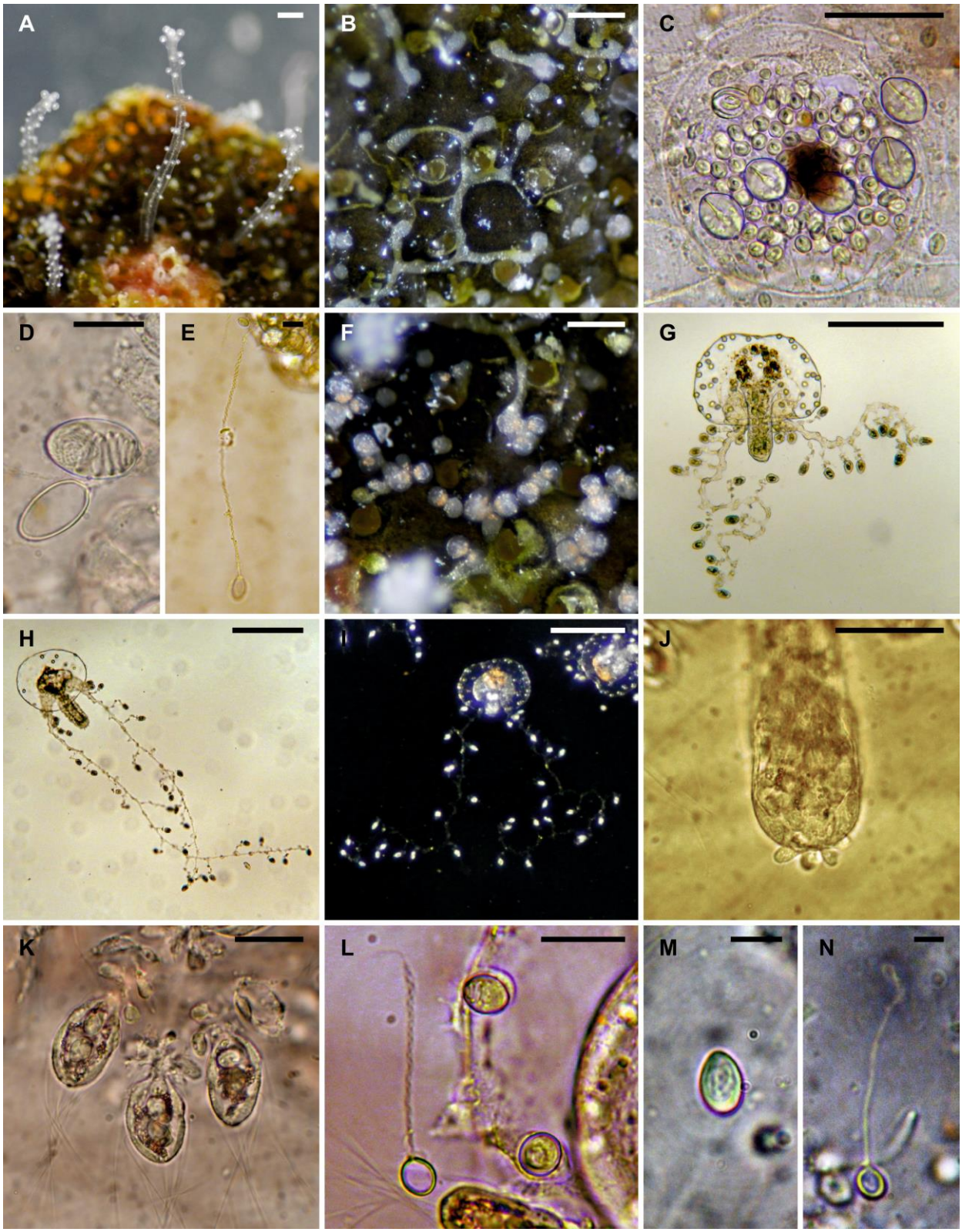

Figure 5.8. *Zanclella labiata*. A) Colony growing in association with the bryozoan host. B) Hydrorhiza partially exposed. C) Capitulum of a tentacle with stenoteles of two size classes. D) Discharged and undscharged capsules of the eurytele type. E) Discharged eurytele. F) Medusa buds arising in clusters directly from the hydrorhiza. G) Two days old medusa. H, I) Five days old medusae. J) Terminal part of the manubrium showing four lips around the mouth. K) Cnidophores containing bean-shaped euryteles. L) Discharged and undischarged capsules of the mastigophores on the exumbrella. M, N) Undischarged and discharged euryteles in the cnidophores. Scale bars: A, B, F-H) 0.2 mm, C, J) 50 μm, D, K, L) 15 μm, M, N) 5 μm.

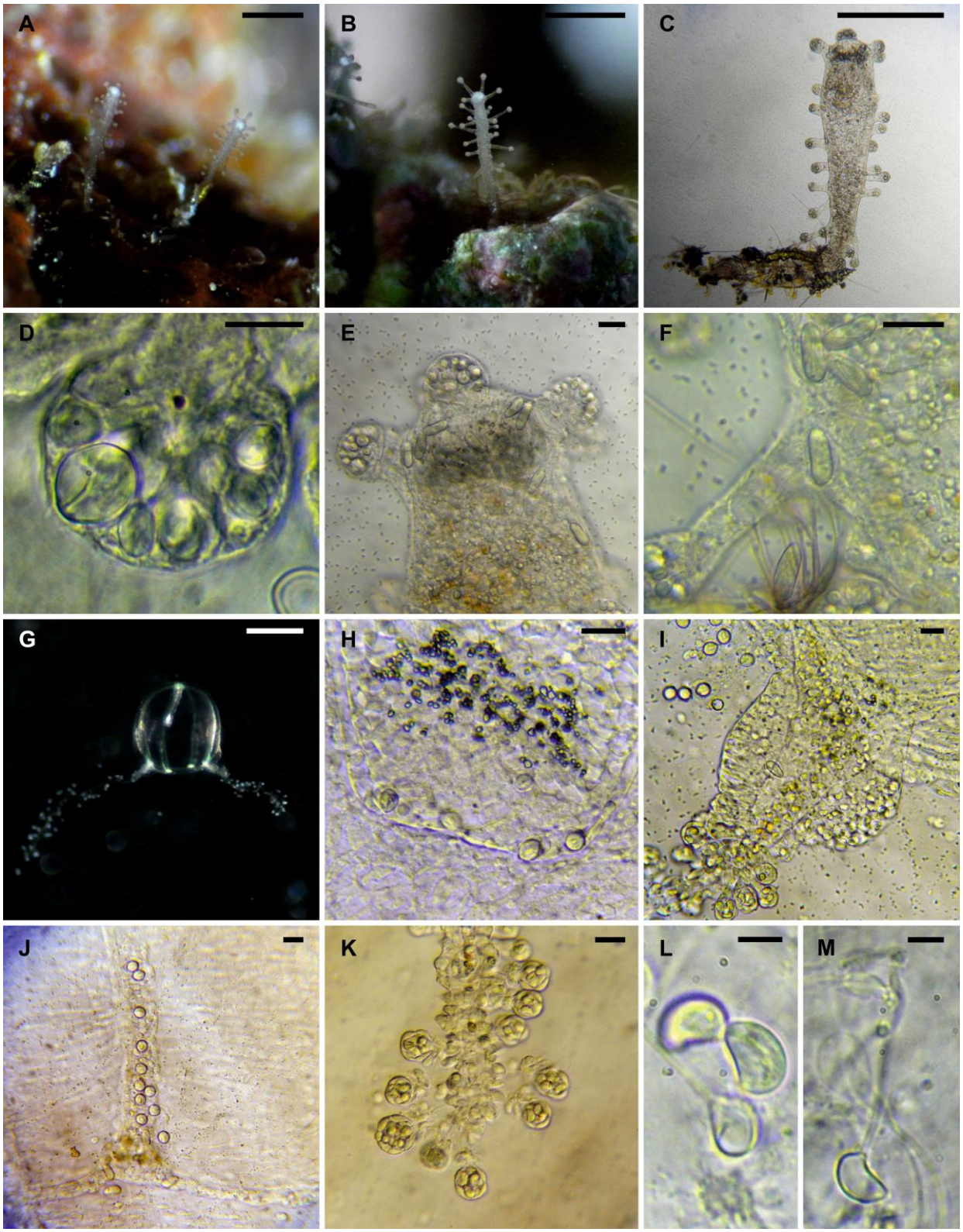

Figure 5.9. *Zanclea* sp. 3. A, B) Polyps growing on a bryozoan and a crustose coralline algae, respectively. C) Detached polyp. D) Capitulum of a tentacle containing stenoteles of two size classes. E, F) Euryteles around the mouth and scattered in the polyp body walls, respectively. G) Newly released medusa. H) Terminal part of the manubrium showing the mouth surrounded by small stenoteles. I) Tentacular bulb with large and small euryteles. J) Exumbrellar nematocyst pouch containing large stenoteles. K) Rounded cnidophores containing bean-shaped euryteles. L, M) Undischarged and discharged euryteles in the cnidophores. Scale bars: A-C, G) 0.5 mm, D-F, H-K) 20 µm, L-M) 5 µm.

CHAPTER 6

Conclusions

6.2. GENERAL CONCLUSIONS

Overall, this work shows that the investigated taxa are much more widespread and diverse than previously known. Indeed, both the geographic distribution and the host range have been widened for several species. For instance, coral-associated hydrozoans were found inhabiting all the investigated tropical localities and new records come from the Indian and Pacific Oceans, Red Sea and Caribbean Sea. These species generally exhibit conserved or little varying morphologies, contrarily to their actual diversity, that has been demonstrated to be high with molecular tools. The use of an integrative taxonomy approach allowed to detect the presence of cryptic species with specific ecological preferences, to find new species and genera, and to assess the phylogenetic position of previously misplaced species. Moreover, the addition of previously unsampled species, as well as the increased number of phylogenetically informative molecular markers, allowed to build robust phylogenetic hypotheses, with increased nodal supports compared to previous studies (e.g. Collins et al. (2005), Nawrocki et al. (2010)). The DNA taxonomy techniques developed in the last years (Fontaneto et al. 2015) are here demonstrated to be efficient tools in species delimitation also for the Zancleida, being able to detect several cryptic species in both coral-associated *Zanclea* and octocorals-associated *Pteroclava*. In particular, scleractinian-associated *Zanclea* can be divided in generalist and genus-specific cryptic species and the hidden diversity of these organisms can be explained by both the host-specificity and the geography. Indeed, most lineages were found specifically associated with a single coral genus, suggesting a role of the hosts or associated organisms in the speciation events (e.g. ecological speciation due to host shift). Additionally, a certain level of isolation of populations from the Red Sea from other populations was detected, and in *Acropora*-associated *Zanclea* this isolation may have led to a speciation event. A similar situation was observed in the *Pteroclava krempfi* species complex. In this group of hydrozoans, four cryptic species are associated with different hosts and these species can be detected with the combination of data on the associated hosts and the geographic distribution, other than the DNA sequences. The use of sequence information in species description (Jörger and Schrödl 2013), as well as a recently proposed 'molecular nomenclature' (Morard et al. 2016), allowed to name the cryptic species found with molecular analyses, facilitating the inclusion of these taxa in further studies, in which they could otherwise be hardly and confusingly considered, and making eventually possible the transfer of knowledge across disciplines (Morard et al. 2016). The integration of morphological and molecular assessments also made possible to detect previously overlooked genera, as well as to reassess the morphological diversity of the analysed families. Specifically, *Zanclea timida* Puce, Di Camillo & Bavestrello 2008 was accommodated into the Cladocorynidae Allman 1872 and included in a new genus, a *Sphaerocoryne*-like species was moved to another newly erected genus, and the new genus and species *Astrocoryne cabela* was discovered. The Zancleidae Russel 1953 were confirmed to be a highly speciose and complex group. This taxon is polyphyletic at both family and genus level and undoubtful morphological diagnoses for the recovered possible new genera and families could not be compiled, due to the conserved and intergrading morphologies. Moreover, a cryptic speciation may have occurred not only in coral-associated *Zanclea*, but also in *Zanclea divergens* Boero, Bouillon & Gravili 2000, which shows a strong

genetic structuring and may be composed of multiple cryptic species. Despite the most comprehensive phylogeny of the family is here presented, many other rare or doubtful species are still missing in molecular analyses and their future inclusion could possibly help in clarifying the taxonomy and systematics of this enigmatic group. The study of the evolution of morphological and ecological characters revealed that a certain level of convergence can be observed both in the Zancleidae and, more generally, in the Zancleida. The mapping of the states of these characters on the recovered phylogenetic hypothesis showed that they are easily lost or regained independently in diverging lineages and that the previously hypothesised evolutionary trends in symbiotic Zancleidae (Boero et al. 2000, Puce et al. 2002) could not be completely confirmed.

To conclude, the results found with this study shed light on different aspects of the investigated taxa and the whole Zancleida superfamily. The integrative approach here used allowed the taxonomic revisions of the three families from the family to the species level and the discovery of previously undetected or overlooked taxa. New information regarding the relationships between these hydrozoans and their host were also provided, enabling further studies devoted at the understanding of the ecology of these associations, as well as of the possible roles of symbiotic hydrozoans in intricate symbiotic systems, such as the coral symbiomes (e.g. Montano et al. (2016), Montano et al. (2017)). Biodiversity loss is currently affecting every ocean and the impact of this trend, for instance, on ocean ecosystem services has been already demonstrated (Worm et al. 2006). Therefore, the understanding of the diversity and ecology of previously overlooked species and the taxonomic composition and interaction of species in complex symbiotic systems, especially those now facing severe declines such as coral reefs (Hughes et al. 2017a, Hughes et al. 2017b), is of high importance and further studies are needed to better understand these fascinating yet endangered species and associations.

6.2. REFERENCES

Boero F, Bouillon J, Gravili C (2000) A survey of *Zanclea*, *Halocoryne* and *Zanclella* (Cnidaria, Hydrozoa, Anthomedusae, Zancleidae) with description of new species. *Italian Journal of Zoology* 67: 93-124.

Collins AG, Winkelmann S, Hadrys H, Schierwater B (2005) Phylogeny of Capitata and Corynidae (Cnidaria, Hydrozoa) in light of mitochondrial 16S rDNA data. *Zoologica Scripta* 34: 91-99.

Fontaneto D, Flot J-F, Tang CQ (2015) Guidelines for DNA taxonomy, with a focus on the meiofauna. *Marine Biodiversity* 45: 433-451.

Hughes TP, Barnes ML, Bellwood DR, Cinner JE, Cumming GS, Jackson JB et al., Morrison TH (2017a) Coral reefs in the Anthropocene. *Nature* 546: 82-90.

Hughes TP, Kerry JT, Álvarez-Noriega M, Álvarez-Romero JG, Anderson KD, Baird AH et al., Berkelmans R (2017b) Global warming and recurrent mass bleaching of corals. *Nature* 543: 373-377.

Jörger KM, Schrödl M (2013) How to describe a cryptic species? Practical challenges of molecular taxonomy. *Frontiers in Zoology* 10: 59.

Montano S, Allevi V, Seveso D, Maggioni D, Galli P (2016) Habitat preferences of the *Pteroclava krempfi*-alcyonaceans symbiosis: inner vs outer coral reefs. *Symbiosis* 72: 225-231.

Montano S, Fattorini S, Parravicini V, Berumen ML, Galli P, Maggioni D, Arrigoni R, Seveso D, Strona G. (2017). Corals hosting symbiotic hydrozoans are less susceptible to predation and disease. *Proceedings of the Royal Society B: Biological Sciences* 284: 20172405.

Morard R, Escarguel G, Weiner AK, André A, Douady CJ, Wade CM et al., Quillévéré F (2016) Nomenclature for the nameless: a proposal for an integrative molecular taxonomy of cryptic diversity exemplified by planktonic foraminifera. *Systematic Biology* 65: 925-940.

Nawrocki AM, Schuchert P, Cartwright P (2010) Phylogenetics and evolution of Capitata (Cnidaria: Hydrozoa), and the systematics of Corynidae. *Zoologica Scripta* 39: 290-304.

Puce S, Cerrano C, Boyer M, Ferretti C, Bavestrello G (2002) *Zanclea* (Cnidaria: Hydrozoa) species from Bunaken Marine Park (Sulawesi Sea, Indonesia). *Journal of the Marine Biological Association of the United Kingdom* 82: 943-954.

Worm B, Barbier EB, Beaumont N, Duffy JE, Folke C, Halpern BS et al., Palumbi SR (2006) Impacts of biodiversity loss on ocean ecosystem services. *Science* 314: 787-790.

APPENDIX

Abstracts of the peer-reviewed papers published during the PhD program

I. PAPERS RELATED TO THE PHD PROJECT

I.1. *Astrocoryne cabela* gen. nov., sp. nov. (Hydrozoa: Sphaerocorynidae), a new sponge-associated hydrozoan

Maggioni Davide, Galli Paolo, Berumen Michael L., Arrigoni Roberto, Seveso Davide, Montano Simone
Invertebrate Systematics (2017) 31: 734-746. doi: 10.1071/IS16091.

The family Sphaerocorynidae includes two valid genera andfive species, most of which have a confusing taxonomic history. Here, a new genus and species, *Astrocoryne cabela*, gen. et sp. nov., is described from the Maldives and the Red Sea, based on both morphological and molecular evidence. *Astrocoryne cabela* has an apomorphy represented by the type of tentacles, here named 'dicapitate', and consisting of capitate tentacles with a proximal capitulum-like cluster of nematocysts. Molecular analyses confirmed the monophyly of this species, as well as its belonging to the Sphaerocorynidae, together with *Sphaerocoryne* spp. and *Heterocoryne caribbensis* Wedler & Larson, 1986, for which we present molecular data for the first time. Moreover, the high divergence of *A. cabela* from other species of the family justifies the establishment of a new genus. Interestingly, specimens from the Maldives and the Red Sea showed marked morphological variation in the polyp stage, although only a slight genetic divergence was detected. This study highlights that a comprehensive morpho-molecular assessment of Sphaerocorynidae is strongly needed in order to clarify the taxonomic issues and the diversity of this taxon.

I.2. Genetic diversity of the *Acropora*-associated hydrozoans: new insight from the Red Sea

Maggioni Davide, Montano Simone, Arrigoni Roberto, Galli Paolo, Puce Stefania, Pica Daniela, Berumen Michael L.
Marine Biodiversity (2017) 47: 1045-1055. doi: 10.1007/s12526-017-0632-4.

To date, four nominal species and several other unidentified species of *Zanclea* hydrozoans are known to live symbiotically with scleractinians, and recent surveys reported this association also in the Red Sea. Previous molecular studies showed that each coral genus involved in this association hosts only one species or molecular clade of *Zanclea*, with the only exception being the genus *Acropora*, which hosts at least two *Zanclea* species. Moreover, some of the detected genetic lineages were morphologically undistinguishable in the polyp stage, suggesting the presence of cryptic species. In this study, we investigated the morphology and genetic diversity of *Acropora*-associated *Zanclea* specimens collected in previous studies in Egypt and Israel, as well as new samples collected in Saudi Arabia. Based on the current data, all the analysed samples were morphologically identical to *Zanclea gallii*, a species associated with *Acropora* corals from the Maldives. However, molecular analyses separated the samples collected in the Red Sea from all other coral-associated hydroids. Therefore, phylogenetic reconstructions, haplotype networks, genetic distance analyses and distribution data allowed us

to identify a previously unknown cryptic species of *Acropora* associated hydroid, here named *Zanclea gallii* IIa, following a recently proposed molecular nomenclature.

I.3. A cryptic species in the *Pteroclava krempfi* species complex (Hydrozoa, Cladocorynidae) revealed in the Caribbean

Montano Simone, Maggioni Davide, Galli Paolo, Hoeksema Bert W.

Marine Biodiversity (2017) 47: 83-89. doi: 10.1007/s12526-016-0555-5.

Symbiotic relationships on coral reefs involving benthic hosts other than scleractinian corals have been poorly investigated. The hydroid *Pteroclava krempfi* is a widespread species known to be mainly associated with alcyonacean octocorals in the Indo-Pacific. In the present study, *P. krempfi* was discovered in association with octocorals of the genus *Antillogorgia* (Gorgoniidae) at two localities in the Caribbean Sea (St. Eustatius in the eastern Caribbean and Bocas del Toro in the western part), updating its host range with an additional genus and family. The Caribbean specimens showed no morphological differences and the shape of their polyps was consistent with the original *P. krempfi* description. A multi-locus phylogeny reconstruction of the *P. krempfi* species complex based on both mitochondrial and nuclear loci revealed three separate molecular clades. Two of them were composed of *P. krempfi* associated with the families Plexauridae and Alcyoniidae from the Maldives, whereas a new highly supported molecular lineage included all Caribbean specimens of *P. krempfi* associated with the family Gorgoniidae. These three divergent molecular clades represent distinct cryptic taxa within the *P.krempfi* species complex, in which the main interspecific difference consists of their host families.

I.4. Molecular evidence for cryptic species in *Pteroclava krempfi* (Hydrozoa, Cladocorynidae) living in association with alcyonaceans

Maggioni Davide, Montano Simone, Seveso Davide, Galli Paolo

Systematics and Biodiversity (2016) 14: 484-493. doi: 10.1080/14772000.2016.1170735.

Hydrozoans are able to establish intimate relationships with several other organisms. The hydroid *Pteroclava krempfi* lives in association with different alcyonacean taxa from Indo-Pacific and Atlantic coral reefs, growing partially embedded within host tissues. In this study, we found *P. krempfi* associated with four alcyonacean hosts from the Maldives, namely *Sinularia*, *Sarcophyton*, *Lobophytum* and *Paraplexaura*, the latter representing a new record. We provided the first molecular phylogenetic evaluation of the genus *Pteroclava*. *Pteroclava krempfi* clustered with *Cladocoryne floccosa*, confirming its position into the family Cladocorynidae. We also performed the first morpho-molecular assessment of *P. krempfi* diversity. All the colonies growing on different hosts revealed polyps and medusae matching published descriptions of *P. krempfi*, showing no morphological differences. However, analysing both nuclear and mitochondrial DNA, two highly supported molecular lineages were identified. These two clades were highly divergent and were specifically associated with hosts belonging to different families (Alcyoniidae and Plexauridae). Therefore,

our results suggest that *P. krempfi* from the Maldives is a complex of cryptic species in which the main diagnostic feature between different species could be the host specificity.

I.5. *Pteroclava krempfi*-octocoral symbiosis: new information from the Indian Ocean and the Red Sea

Seveso Davide, Montano Simone, Pica Daniela, Maggioni Davide, Galli Paolo, Allevi Virginia, Bastari Azzurra, Puce Stefania

Marine Biodiversity (2016) 46: 483-487. doi: 10.1007/s12526-015-0368-y.

Some hydroids are known to form strict relationships with anthozoans. In this study we report the first evidence of the association between *Pteroclava krempfi* (Hydrozoa: Capitata: Cladocorynidae) and octocorals of the alcyonacean genera *Sarcophyton*, *Lobophytum* and *Sinularia* observed in the Republic of Maldives and in the Red Sea. Our observations contribute to an expansion of both the recorded host-range and geographical distribution of this symbiosis, indicating that the association between hydroids and alcyonaceans is more widespread than previously known.

I.6. The hidden diversity of *Zanclea* associated with scleractinians revealed by molecular data

Montano Simone, Maggioni Davide, Arrigoni Roberto, Seveso Davide, Puce Stefania, Galli Paolo

PloS one (2015) 10: e0133084. doi: 10.1371/journal.pone.0133084.

Scleractinian reef corals have recently been acknowledged as the most numerous host group found in association with hydroids belonging to the *Zanclea* genus. However, knowledge of the molecular phylogenetic relationships among *Zanclea* species associated with scleractinians is just beginning. This study, using the nuclear 28S rDNA region and the fast-evolving mitochondrial 16S rRNA and COI genes, provides the most comprehensive phylogenetic reconstruction of the genus *Zanclea* with a particular focus on the genetic diversity among *Zanclea* specimens associated with 13 scleractinian genera. The monophyly of *Zanclea* associated with scleractinians was strongly supported in all nuclear and mitochondrial phylogenetic reconstructions. Furthermore, a combined mitochondrial 16S and COI phylogenetic tree revealed a multitude of hidden molecular lineages within this group (Clades I, II, III, V, VI, VII, and VIII), suggesting the existence of both host-generalist and genus-specific lineages of *Zanclea* associated with scleractinians. In addition to *Z. gallii* living in association with the genus *Acropora*, we discovered four well-supported lineages (Clades I, II, III, and VII), each one forming a strict association with a single scleractinian genus, including sequences of *Zanclea* associated with *Montipora* from two geographically separated areas (Maldives and Taiwan). Two host-generalist *Zanclea* lineages were also observed, and one of them was formed by *Zanclea* specimens symbiotic with seven scleractinian genera (Clade VIII). We also found that the COI gene allows the recognition of separated hidden lineages in agreement with the commonly recommended mitochondrial 16S as a DNA barcoding gene for Hydrozoa and shows reasonable potential for phylogenetic and evolutionary analyses in

the genus *Zanclea*. Finally, as no DNA sequences are available for the majority of the nominal *Zanclea* species known, we note that they will be necessary to elucidate the diversity of the *Zanclea*-scleractinian association.

II. OTHER PAPERS

II.1. A reassessment of *Halopteris polymorpha* (Billard, 1913) (Cnidaria: Hydrozoa), with descriptions of three new species

Galea Horia R., Di Camillo Cristina G., Maggioni Davide, Montano Simone, Schuchert Peter

Revue Suisse de Zoologie (2018) 125: 1-39.

Several hydroids, corresponding to various morphotypes included earlier in the synonymy of *Halopteris polymorpha* (Billard, 1913), occur in materials obtained recently from Indonesia and the Maldives, or are housed in the collection of the Muséum d'Histoire Naturelle of Geneva, Switzerland. Among them, new specimens, indistinguishable morphologically from the lectotype, are fully redescribed, together with the so-called variety *sibogae* Billard, 1913. While the latter displays in life an original, not yet documented coloration (bright yellow cauline polyps contrasting with their pure white cladial counterparts), the former is uniformly yellow throughout. This feature, combined with a series of morphological differences, demonstrates that we are dealing with a well-characterized species, whose name should be *H. sibogae* (Billard, 1913). The so far unknown gonothecae of the latter are described for the first time, together with the males of the nominal species. The taxonomy of *H. polymorpha* is analyzed in-depth and reassessed, where available also using 16S DNA sequences. Morphological traits can be used to split the species complex and allow the separation of three as yet undescribed species, *H. australis* from New Caledonia and French Polynesia, *H. millardae* from the Maldives and the Seychelles, and *H. brasiliensis* from Brazil. Additionally, new records of *H. vervoorti* Galea, 2008 extend its known geographical distribution to Madagascar, the Maldives and Indonesia, while some literature records suggest that it could spread as far as Australia, Japan and Fiji. All species are fully described and illustrated, and their morphology is compared to that of their related congeners.

II.2. The zoogeography of extant rhabdopleurid hemichordates (Pterobranchia : Graptolithina), with a new species from the Mediterranean Sea

Beli Elena, Aglieri Giorgio, Strano Francesca, Maggioni Davide, Telford Max J., Piraino Stefano, cameron Christopher B.

Invertebrate Systematics (2018) 32: 100-110. doi:10.1071/IS17021.

The early origin and evolutionary radiation of graptolites (Hemichordata: Pterobranchia) is a story told almost entirely in the fossil record, but for four extant species of the genus *Rhabdopleura* Allman, 1869. Here we report the discovery of a fifth species, *Rhabdopleura recondita*, sp. nov., at a depth range of 2–70 m from the Adriatic and Ionian Seas, always associated with bryozoans in coralligenous habitats. This is the first

pterobranch record in Italian waters, and the second in the Mediterranean Sea. The new species is characterised by: (1) tubaria with smooth creeping tubes adherent to the inside of empty bryozoan zooecia; (2) erect outer tubes with a graptolite, fusellar-like organisation; and (3) zooids that extend from a black stolon, which is free from the creeping tube. Each of the paired feeding arms has two rows of tentacles that do not extend to the arm tip. The distal ends of the arms, the collar and the cephalic shield are replete with black granules. Phylogenetic analyses of individual and concatenated gene sequences of mitochondrial *16SrDNA* and nuclear *18SrDNA* support the validity of *R. recondita* as a new species. Finally, we discuss the global biogeographic and habitat distributions of the extant *Rhabdopleura* representatives.

II.3. Corals hosting symbiotic hydrozoans are less susceptible to predation and disease

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In spite of growing evidence that climate change may dramatically affect networks of interacting species, whether - and to what extent - ecological interactions can mediate species' responses to disturbances is an open question. Here we show how a largely overseen association such as that between hydrozoans and scleractinian corals could be possibly associated with a reduction in coral susceptibility to ever-increasing predator and disease outbreaks. We examined 2455 scleractinian colonies (from both Maldivian and the Saudi Arabian coral reefs) searching for non-random patterns in the occurrence of hydrozoans on corals showing signs of different health conditions (i.e. bleaching, algal overgrowth, corallivory and different coral diseases). We show that, after accounting for geographical, ecological and co-evolutionary factors, signs of disease and corallivory are significantly lower in coral colonies hosting hydrozoans than in hydrozoan-free ones. This finding has important implications for our understanding of the ecology of coral reefs, and for their conservation in the current scenario of global change, because it suggests that symbiotic hydrozoans may play an active role in protecting their scleractinian hosts from stresses induced by warming water temperatures.

II.4. Camouflage of sea spiders (Arthropoda, Pycnogonida) inhabiting *Pavona varians*

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Despite the controversial phylogenetic position of the Class Pycnogonida, sea spiders account for an enormous diversity of species, inhabiting all benthic marine habitats worldwide. Although they have been observed on coral reefs, few sea spider species have been reported in association with coral reef organisms and even more rarely with reef-building corals. In a biodiversity study in 2017, various individuals of *Endeis* sp. were observed on *Pavona varians* colonies inhabiting the coral reefs surrounding Magoodhoo Island, Maldives. The sea spiders were generally observed dwelling on and between the corallites of *P. varians*, with each leg

overlapping the natural long septa of the coral. Their motionless behaviour rendered them completely undetectable without careful inspection. The nature of the relationship between the corals and sea spiders on this reef is not clear, although Arango (2001) previously reported feeding behaviour by sea spiders on fire corals. Moreover, sea spiders are also known to be parasitically associated with several organisms, including at least 20 hydrozoan species, in which the gastrovascular cavities of the polyps are used for the development of the pycnogonid protonymphs. It is likely that *Pavona*-inhabiting sea spiders, as well as other coral cryptobenthic associates, are commensals, just looking for shelter among corallites or coral tentacles; however, the co-occurrence of coral-associated hydrozoans of the genus *Zanclea* and egg-carrying individuals on all observed *P. varians* colonies suggests many possible scenarios. There have been few research efforts investigating small coral-associated invertebrates with extremely successful cryptic strategies, such as these pycnogonids, and it is likely that this has led to the lack of observations regarding this association. To our knowledge, this finding represents the first record of sea spiders observed on reef-building corals in the Indian Ocean and serves to improve the scarce knowledge of coral-associated sea spiders.

II.5. Description of *Turritopsoides marhei* sp. nov. (Hydrozoa, Anthoathecata) from the Maldives and its phylogenetic position

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Turritopsoides marhei, a new species of the hydrozoan family Oceaniidae, is described from the Maldives. This species can be distinguished from the only other member of the genus by the presence of more branched colonies, branches not being adnate to pedicels, longer pedicels, larger nematocysts, nematocyst-rich nematophore-like outgrowths from pedicels, smaller male gonophores, and a different geographic distribution. This finding represents the first record of the genus outside the type locality of its type species, in Belize. Molecular phylogenetic analyses show that, as expected, *T. marhei* belongs to the clade Filifera IV. However, the phylogenetic hypothesis based on both mitochondrial and nuclear DNA sequences reveals that most of the families of this group are polyphyletic, including Oceaniidae, and suggests that the morphological characters used to discriminate among filiferan families need to be revised thoroughly.

II.6. The cellular stress response of the scleractinian coral *Goniopora columna* during the progression of the Black band disease

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Black band disease (BBD) is a widespread coral pathology caused by a microbial consortium dominated by cyanobacteria, which is significantly contributing to the loss of coral cover and diversity worldwide. Since the

effects of the BBD pathogens on the physiology and cellular stress response of coral polyps appear almost unknown, the expression of some molecular biomarkers, such as Hsp70, Hsp60, HO-1, and MnSOD, was analyzed in the apparently healthy tissues of *Goniopora columna* located at different distances from the infection and during two disease development stages. All the biomarkers displayed different levels of expression between healthy and diseased colonies. In the healthy corals, low basal levels were found stable over time in different parts of the same colony. On the contrary, in the diseased colonies, a strong up-regulation of all the biomarkers was observed in all the tissues surrounding the infection, which suffered an oxidative stress probably generated by the alternation, at the progression front of the disease, of conditions of oxygen supersaturation and hypoxia/anoxia, and by the production of the cyanotoxin microcystin by the BBD cyanobacteria. Furthermore, in the infected colonies, the expression of all the biomarkers appeared significantly affected by the development stage of the disease. In conclusion, our approach may constitute a useful diagnostic tool, since the cellular stress response of corals is activated before the pathogens colonize the tissues, and expands the current knowledge of the mechanisms controlling the host responses to infection in corals.

II.7. Habitat preferences of the *Pteroclava krempfi*-alcyonaceans symbiosis: inner vs outer coral reefs

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Herein, we provide observation on the ecological relationships between the hydrozoan species *Pteroclava krempfi* and three alcyonacean genera: *Lobophytum*, *Sarcophyton* and *Sinularia* from protected and exposed reef habitats in the Maldives. The associations were found to be widespread in the investigated area with both an overall and taxon-specific symbiosis prevalence higher in the exposed reef sites. *Pteroclava krempfi* most frequently occurred with *Lobophytum*, followed by *Sinularia* and *Sarcophyton*. The prevalence of *P. krempfi* with soft corals was also positively correlated to percent host cover, which was higher in the outer reef sites, suggesting a host-reliant relationship for the hydrozoan. However, the nature of these relationships, as well as the factors that drive their establishment, requires further investigation. The widespread degradation of coral reef ecosystems endangers the existence of many poorly understood, but intimate relationships that often go unrecognized.

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