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Green Fluorescence Patterns in Closely Related Symbiotic Species of *Zanclea* (Hydrozoa, Capitata)

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Abstract: Green fluorescence is a common phenomenon in marine invertebrates and is caused by green fluorescent proteins. Many hydrozoan species display fluorescence in their polyps and/or medusa stages, and in a few cases patterns of green fluorescence have been demonstrated to differ between closely related species. Hydrozoans are often characterized by the presence of cryptic species, due to the paucity of available morphological diagnostic characters. *Zanclea* species are not an exception, showing high genetic divergence compared to a uniform morphology. In this work, the presence of green fluorescence and the morpho-molecular diversity of six coral- and bryozoan-associated *Zanclea* species from the Maldivian coral reefs were investigated. Specifically, the presence of green fluorescence in polyps and newly released medusae was explored, the general morphology, as well as the cnidome and the interaction with the hosts, were characterized, and the *16S rRNA* region was sequenced and analyzed. Overall, *Zanclea* species showed a similar morphology, with little differences in the general morphological features and in the cnidome. Three of the analyzed species did not show any fluorescence in both life stages. Three other *Zanclea* species, including two coral-associated cryptic species, were distinguished by species-specific fluorescence patterns in the medusae. Altogether, the results confirmed the morphological similarity despite high genetic divergence in *Zanclea* species and indicated that fluorescence patterns may be a promising tool in further discriminating closely related and cryptic species. Therefore, the assessment of fluorescence at a large scale in the whole Zancleidae family may be useful to shed light on the diversity of this enigmatic taxon.

Keywords: integrative taxonomy; symbiosis; corals; bryozoans; Maldives; phylogeny

1. Introduction

Green fluorescence is a diffuse phenomenon in the marine environment, being found in a variety of taxa, including cnidarians, ctenophores, crustaceans, and chordates [1]. Green fluorescence is caused by green fluorescence proteins, which were firstly described in the hydrozoan species *Aequorea victoria* (Murbach and Shearer, 1902) [2]. Lately, similar proteins were detected in several other species, mainly belonging to the Anthozoa [3], and they are currently known to be widespread in the marine metazoans [4]. In most cases, the ecological function of fluorescence is still unclear, even though some hypotheses have been proposed. For instance, in anthozoans associated with

unicellular algae, fluorescent proteins may have a role in regulating the light environment of the symbionts [5], whereas in bioluminescent organisms they seem to be involved in the modification of bioluminescence emission [6]. However, these hypotheses do not apply to non-symbiotic (with algae) and non-bioluminescent species. Other possible roles of fluorescence in marine organisms relate to camouflage, intraspecific communication [7], and prey attraction [8,9]. The latter hypothesis seems to fit better for hydrozoans, since it has been experimentally demonstrated that at least one species, *Olindias formosus* (Goto, 1903), uses fluorescence in tentacles to attract juvenile fish preys [8].

Among hydrozoans, green fluorescence is common and has been reported from polyps and medusae of several species (see [10] and references therein). In medusae, fluorescence is found in the umbrella, radial and circular canals, manubrium, gonads, bulbs, and tentacles (e.g., [11–13]), whereas in polyps in the hydrocaulus, hypostome, and in the epithelium below tentacles [10,13,14]. Green fluorescence patterns were found to differ significantly in closely related species of *Eugymnanthea* Palombi, 1936 [11], and even if these patterns changed during the development, they remained distinguishable from those in the relatives [11]. Moreover, Prudkovsky et al. [10] recently demonstrated that these patterns also differ between cryptic or pseudo-cryptic species of *Cytaeis* Eschscholtz, 1829, indicating that they may be reliable and informative taxonomic characters that could be useful especially when dealing with morphologically undistinguishable species.

Indeed, cryptic species are common in hydrozoans, since morphologically very similar polyps and medusae often show strong genetic diversification, that in many cases relates to host specialization and geography (e.g., [15–17]). This is especially true for the capitate family Zancleidae Russel, 1953, in which the few morphological diagnostic characters available make species identification and description challenging [18,19]. The cnidome is considered a useful character to discriminate among zancleid species, due to the variation of type and size of nematocysts in different species [20]. For instance, the statistical treatment of nematocysts measurements of three *Zanclea* cryptic species resulted in significant differences between the taxa [21], further supporting the importance of the cnidome as a reliable taxonomic character. Another useful character to distinguish closely related symbiotic species is the host specificity, since some species or lineages are specifically associated with one or a few invertebrate taxa (e.g., scleractinian corals) [16,18]. Moreover, some coral-associated *Zanclea* species were found to induce modifications of the host skeletons that could be taxonomically informative [21].

In this work we analyzed the morphology (polyps, newly released medusae, and modifications of the hosts) and genetic diversity (*16S rRNA*) of six symbiotic *Zanclea* species collected in the Maldives. Yet, along with the morpho-molecular analyses, we investigated the informativeness of green fluorescence patterns of polyps and medusae to discriminate between closely related taxa.

2. Materials and Methods

2.1. Morphological Analyses and Fluorescence Essay

Colonies of symbiotic *Zanclea* species were collected in reefs around Magoodhoo Island, Faafu Atoll, Republic of the Maldives (3.0782° N, 72.9613° E), during February 2017. Six *Zanclea* species were collected: *Zanclea sango* Hirose and Hirose, 2011 and *Zanclea* sp. (Clade I, *sensu* [18]) associated with the scleractinians *Pavona varians* (Verril, 1864) and *Goniastrea* sp., respectively; *Zanclea divergens* (Boero, Bouillon, and Gravili, 2000), *Zanclea* sp. 1 and *Zanclea* sp. 2 (*sensu* [22]) associated with the bryozoans *Celleporaria vermiformis* (Waters, 1909), *Celleporaria pigmentaria* (Waters, 1909), and *Celleporaria* sp., respectively; *Zanclea* cf. *protecta* associated with the bryozoans *Parasmittina* cf. *spondylicola* and *Schizoporella* sp. For comparison, *Asyncoryne ryniensis* Warren, 1908 was included in the analyses, since it is closely related to the family Zancleidae [22]. For each *Zanclea* species three colonies were collected, whereas two colonies of *A. ryniensis* were analyzed, for a total of 20 samples. Hydrozoan colonies were collected together with their hosts using hammer and chisel, by snorkeling or SCUBA diving. Colonies were immediately transferred in bowls with seawater after diving, and they were kept in the laboratories of the Marine Research and High Education (MaRHE) Center in Magoodhoo. One

colony per species had medusa buds at the time of sampling, and these colonies were reared until medusae were released. Seawater was replaced daily, approximately two hours after a feeding session with *Artemia* nauplii. Newly released medusae were reared for a few days and then anesthetized with menthol crystals and fixed with 10% formalin for further morphological analyses. Hydrozoan polyps were detached from their hosts using precision forceps and micropipettes, and they were fixed in 10% formalin and 99% ethanol for morphological and genetic analyses, respectively. Formalin-preserved polyps and medusae were analyzed using a Zeiss Axioskop 40 compound microscope to observe their general morphology and characterize their cnidome. Measurements were taken using the software ImageJ 1.52p. All pictures were taken using Canon G7X Mark II camera.

To investigate possible modifications related to the associations with hydroids, the skeletons of the hosts were analyzed under a scanning electron microscope. Specifically, fragments of the *Zanclea*-bearing bryozoan and scleractinian colonies were immersed in a 10% sodium hypochlorite solution for 6–24 h. After rinsing, fragments were sputter-coated with gold and observed under a Zeiss Gemini SEM 500 scanning electron microscope.

Before fixation, all hydrozoan polyps (n = 15 for each species and colony) and medusae (n = 5–15 for each species) were checked for green fluorescence emission using a Leica EZ4 D stereomicroscope equipped with a Weefine Smart Focus 2300 lamp (excitation wavelength: 420 nm) and yellow filter. All medusae were observed at day one and five after release.

2.2. Molecular Characterization

Genetic analyses were performed to check the molecular identity of the samples (n = 20) and to assess their phylogenetic relationships. DNA was extracted from one polyp per colony using a protocol modified from Zietara et al. [23] and already used proficiently to extract DNA from hydrozoans (e.g., [24]). A portion of the *16S rRNA* was then amplified using the primers and protocol described in Cunningham and Buss [25]. The success of PCRs was assessed through an electrophoretic run in 1% agarose gel. PCR products were purified and sequenced in forward and reverse directions with the same primers used for amplification, with ABI 3730xl DNA Analyzer (Applied Biosystems). The obtained chromatograms were visually checked and assembled using Geneious 6.1.6 and sequences were deposited with the EMBL (GenBank accession numbers: MN923260-MN923279). Each sequence was searched in the NCBI BLASTn database to confirm the morphological identifications. All the obtained sequences were then aligned using MAFFT 7.110 [26], with the *E-INS-i* option and the sequences of *Cladocoryne haddoni* and *Pennaria disticha* (GenBank accession numbers: MG811591 and LT746002, respectively) were included as outgroups. The best-fitting evolutionary model was determined using JModelTest 2 [27] and resulted in GTR+I+G, following the Akaike Information Criterion. Phylogenetic trees were built using both Bayesian inference and maximum likelihood approaches. For Bayesian analyses, MrBayes 3.2.6 [28] was used, and four parallel Markov Chain Monte Carlo runs (MCMC) were run for 10^7 generations, trees were sampled every 1000^{th} generation, and burn-in was set to 25%. Maximum likelihood trees were built with RAxML 8.2.9 [29] using 1000 bootstrap replicates.

Pairwise genetic distances between and within species were calculated as % uncorrected p-distances with 1000 bootstrap replicates using MEGA X [30].

3. Results

3.1. General Morphology of Polyps and Medusae

All the analyzed *Zanclea* species showed a similar morphology in both polyp and medusa stages (Figures 1–6). All polyps were colonial, cylindrical, or claviform, with a whorl of oral capitate tentacles and aboral tentacles scattered on the hydranth body wall. Bryozoan-associated species (*Zanclea divergens*, *Zanclea* cf. *protecta*, *Zanclea* sp. 1, and *Zanclea* sp. 2) were monomorphic and deprived of perisarc, whereas the scleractinian-associated *Zanclea sango* and *Zanclea* sp. (Clade I) showed polymorphic polyps, having both gastrozooids and dactylozooids, and the hydrorhiza was surrounded

by a thin layer of chitinous perisarc. All species had stenotele capsules in their capitula, and apart from *Zanclea* cf. *protecta*, all had euryteles in their polyps and/or hydrorhiza. Medusa buds arose directly from the hydrorhiza in *Zanclea divergens*, *Zanclea* sp. 1, and *Zanclea* sp. 2, whereas they were borne on both gastrozooids and hydrorhiza in *Zanclea* cf. *protecta*, *Zanclea sango*, and *Zanclea* sp. (Clade I). Medusae had a bell-shaped or globular umbrella, with nematocysts scattered over the surface in all species apart from scleractinian-associated species. *Zanclea* sp. 1 and *Zanclea* sp. 2 did not have canals and exumbrellar nematocyst pouches at release, whereas all other species had four radial and one circular canal and four nematocyst pouches containing stenoteles and euryteles (the latter only in coral-associated species). Manubria were cylindrical and had stenoteles around the mouth in *Z. divergens*, *Z.* cf. *protecta*, *Zanclea sango*, and *Zanclea* sp. (Clade I). *Zanclea* sp. 1 and *Zanclea* sp. 2 had no nematocysts on the manubrium but four short oral arms. All medusae had two opposite tentacles, bearing a variable number of rounded or elongated cnidophores containing bean-shaped macrobasic euryteles.

Asyncoryne ryniensis (Figure 7) polyps had a distinct morphology, being characterized by a whorl of capitate oral tentacles and moniliform tentacles scattered on the hydranth body wall. Polyps were monomorphic and had both stenoteles and euryteles. Medusa buds were borne on the distal half of polyps. The medusa stage was very similar to that of *Zanclea* species, showing a bell-shaped umbrella, one circular and four radial canals, four exumbrellar nematocyst pouches, four bulbs, and two opposite tentacles bearing cnidophores with macrobasic euryteles inside.

Detailed characterizations of morphology and cnidome of polyps and medusae of all species are summarized in Tables 1 and 2.

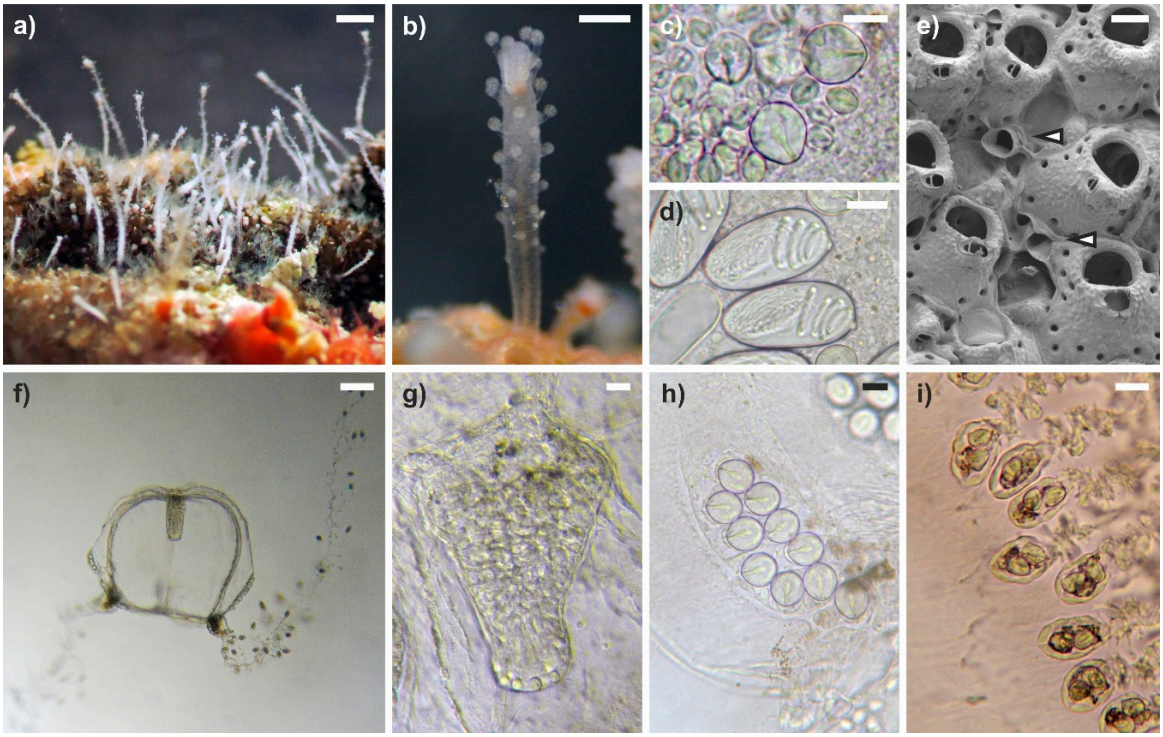

Figure 1. *Zanclea divergens*. (**a**) Colony associated with *Celleporaria vermiformis*; (**b**) close-up of a polyp; (**c**) stenoteles in the capitula, and (**d**) euryteles in the hypostome; (**e**) tube-like skeletal modifications of the bryozoan skeleton (arrowheads); (**f**) newly released medusa and close-up of (**g**) manubrium, (**h**) nematocyst pouch, and (**i**) cnidophores. Scale bars: (**a**) 0.5 mm; (**b**,**e**,**f**) 0.1 mm; (**c**,**d**,**g**–**i**) 10 µm.

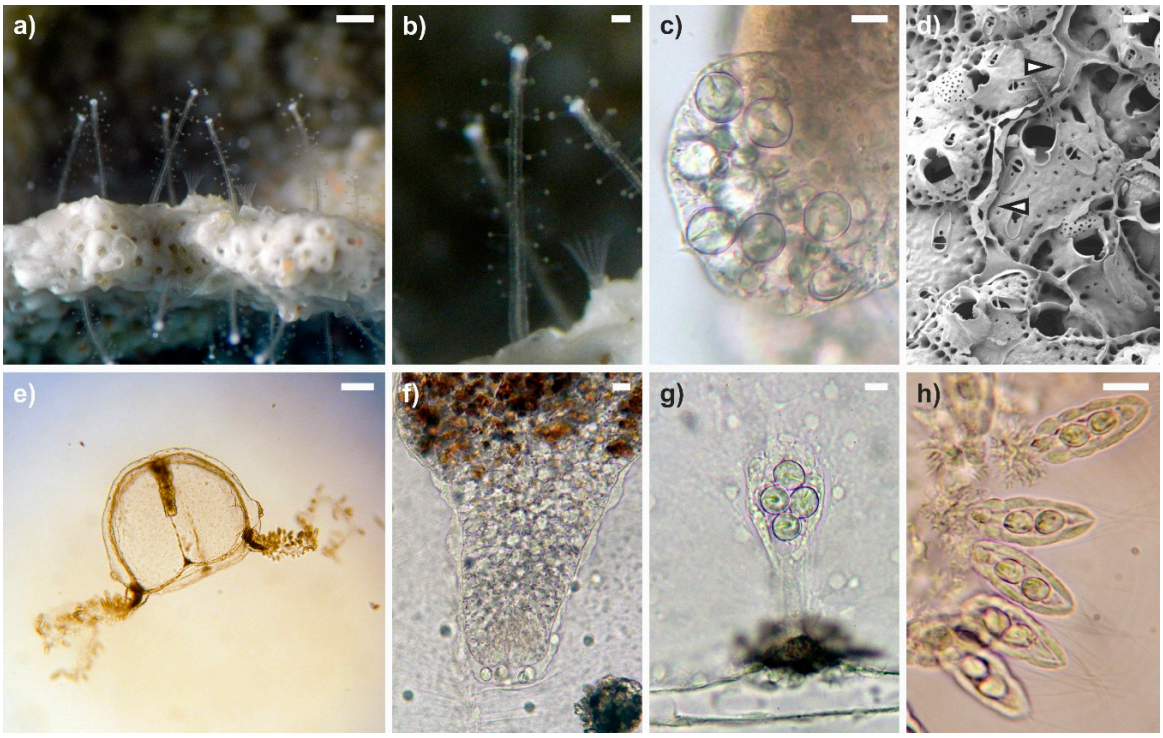

Figure 2. *Zanclea* cf. *protecta*. (**a**) Colony associated with *Parasmittina* cf. *spondylicola*; (**b**) close-up of a polyp; (**c**) stenoteles in the capitula; (**d**) bryozoan skeletal lamina overgrowing the hydrorhiza (arrowheads); (**e**) newly released medusa; close-ups of (**f**) manubrium, (**g**) nematocyst pouch, and (**h**) cnidophores. Scale bars: (**a**) 0.5 mm; (**b**,**d**,**e**) 0,1 mm; (**c**,**f**–**h**) 10 μm.

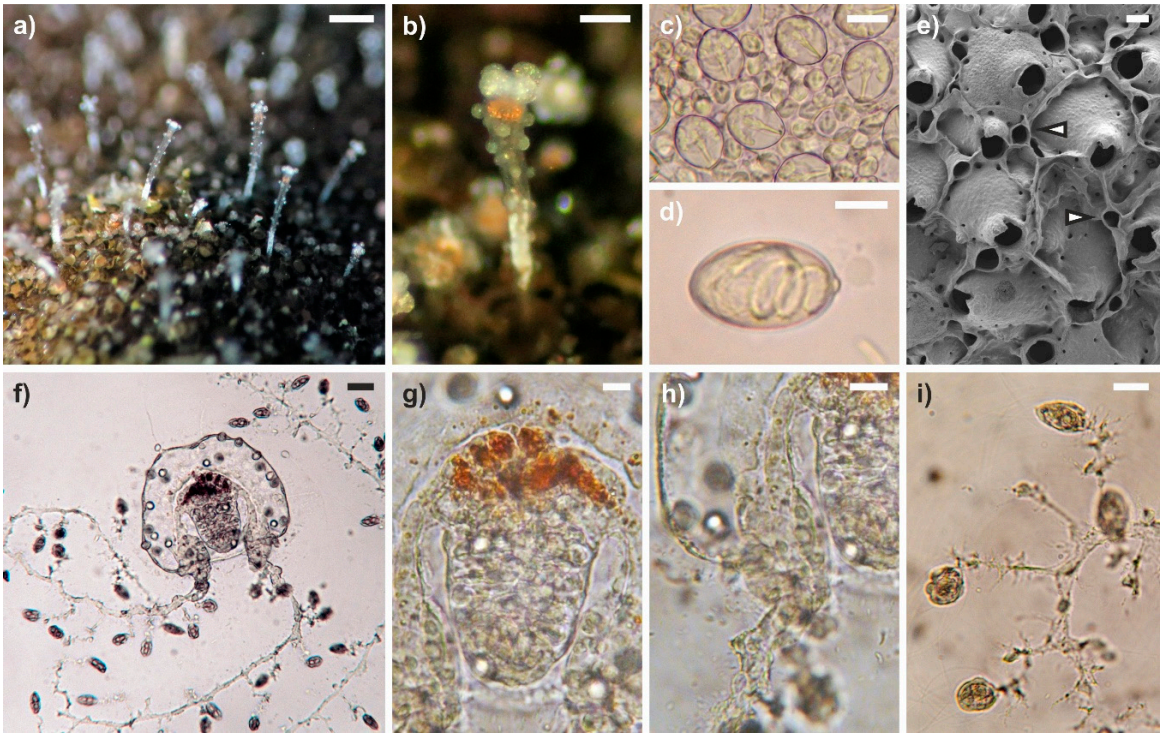

Figure 3. *Zanclea* sp. 1. (**a**) Colony associated with *Celleporaria pigmentaria*; (**b**) close-up of a polyp; (**c**) stenoteles in the capitula, and (**d**) eurytele in the hydrorhiza; (**e**) tube-like modifications of the bryozoan skeleton (arrowheads); (**f**) newly released medusa; close-ups of (**g**) manubrium, (**h**) tentacular bulb, and (**i**) cnidophores. Scale bars: (**a**) 0.5 mm; (**b**,**e**) 0.1 mm; (**c**,**d**,**g**,**h**) 10 μm; (**f**) 20 μm.

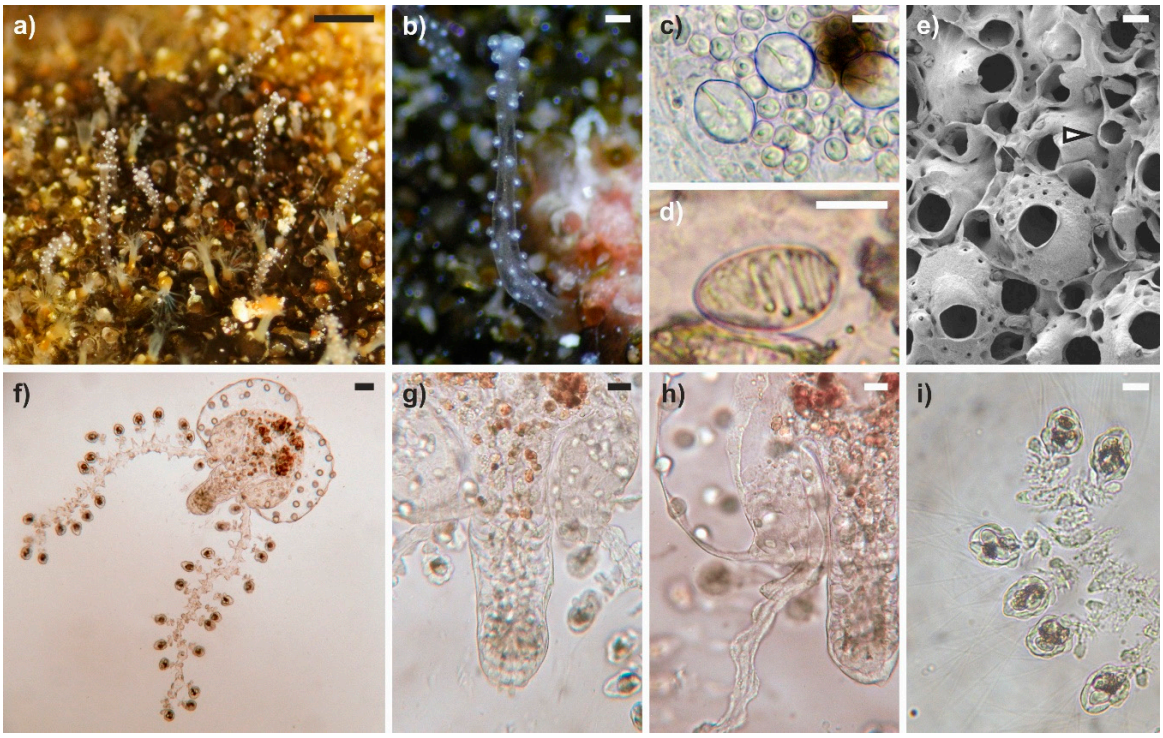

Figure 4. *Zanclea* sp. 2. (**a**) Colony associated with *Celleporaria* sp.; (**b**) close-up of a polyp; (**c**) stenoteles in the capitula, and (**d**) euryteles in the hydrorhiza; (**e**) tube-like modifications of the bryozoan skeleton (arrowheads); (**f**) newly released medusa; close-ups of (**g**) manubrium, (**h**) tentacular bulb, and (**i**) cnidophores. Scale bars: (**a**) 0.5 mm; (**b**,**e**) 0.1 mm; (**c**,**d**,**g**–**i**) 10 μm; (**f**) 20 μm.

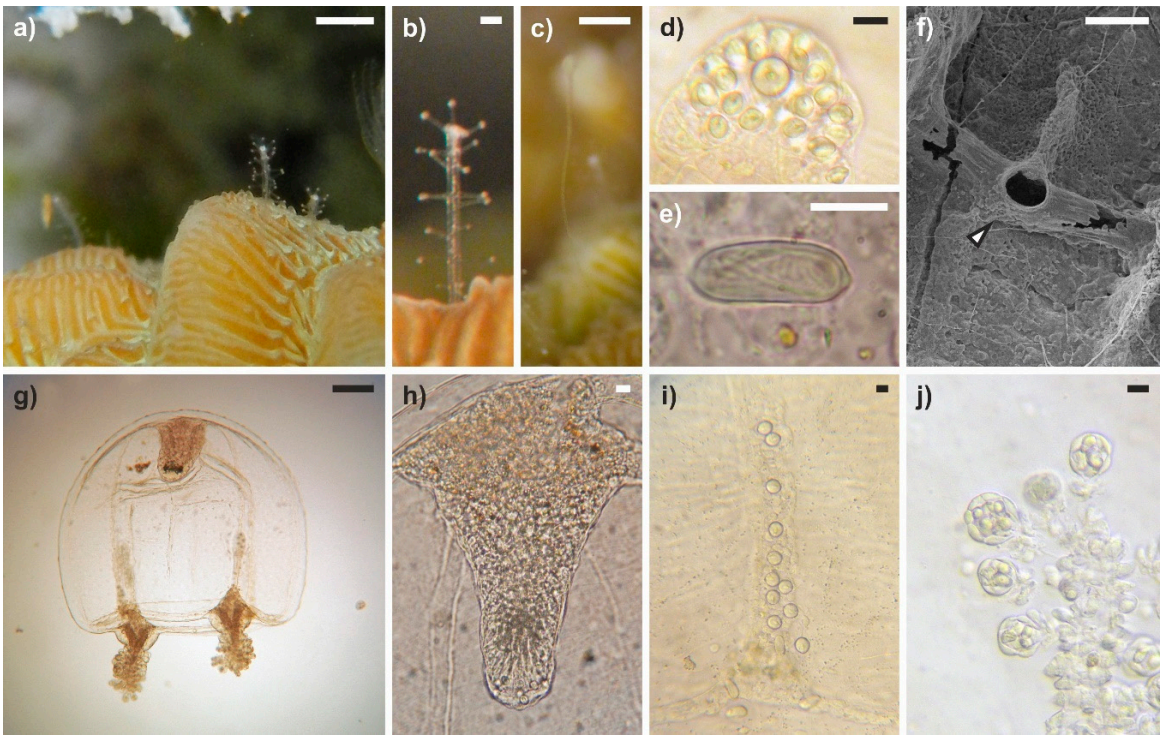

Figure 5. *Zanclea sango*. (**a**) Colony associated with *Pavona varians*; close-ups of (**b**) gastrozooid, and (**c**) dactylozooid; (**d**) stenoteles in the capitula, and (**e**) eurytele in the hypostome; (**f**) micro-alteration of the coral skeleton (arrowhead); (**g**) newly released medusa; close-ups of (**h**) manubrium, (**i**) nematocyst pouch, and (**j**) cnidophores. Scale bars: (**a**,**c**) 0.5 mm; (**b**,**f**,**g**) 0.1 mm; (**d**,**e**,**h**–**j**) 10 μm.

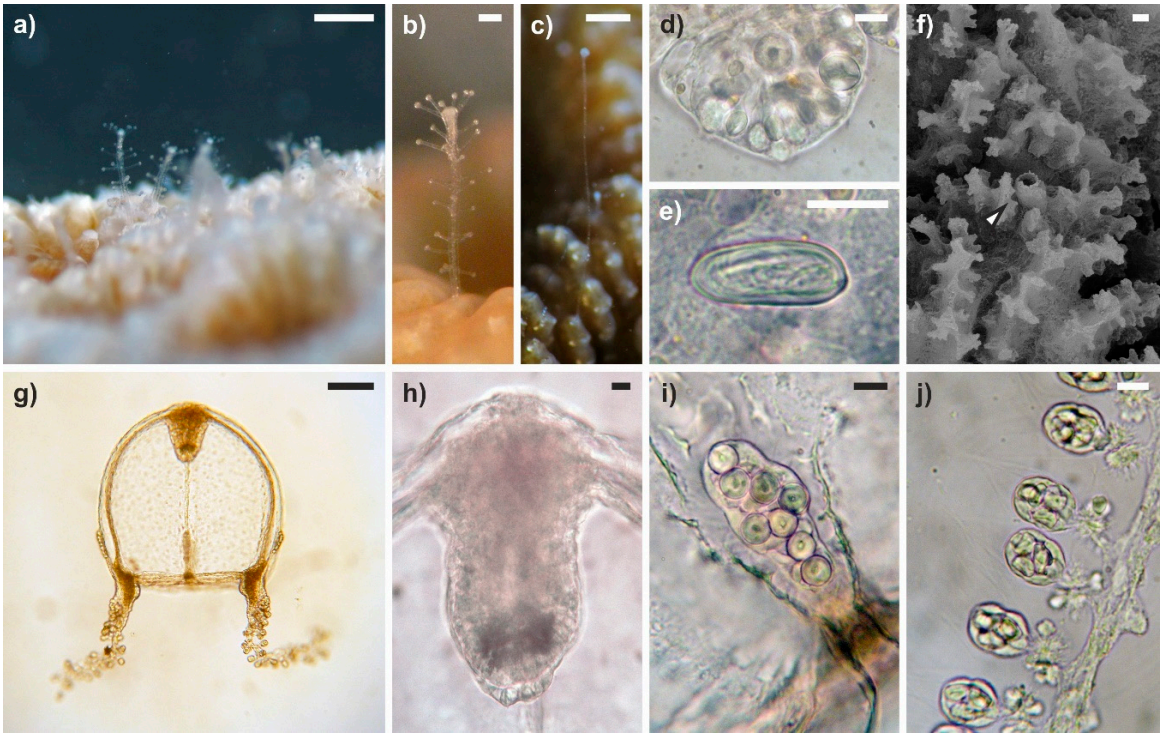

Figure 6. *Zanclea* sp. (Clade I). (**a**) Colony associated with *Goniastrea* sp.; close-ups of (**b**) gastrozooid and (**c**) dactylozooid; (**d**) stenoteles in the capitula, and (**e**) eurytele in the hypostome; (**f**) micro-alteration of the coral skeleton (arrowhead); (**g**) newly released medusa; close-ups of (**h**) manubrium, (**i**) nematocyst pouch, and (**j**) cnidophores. Scale bars: (**a**,**c**) 0.5 mm; (**b**,**f**,**g**) 0.1 mm; (**d**,**e**,**h**–**j**) 10 µm.

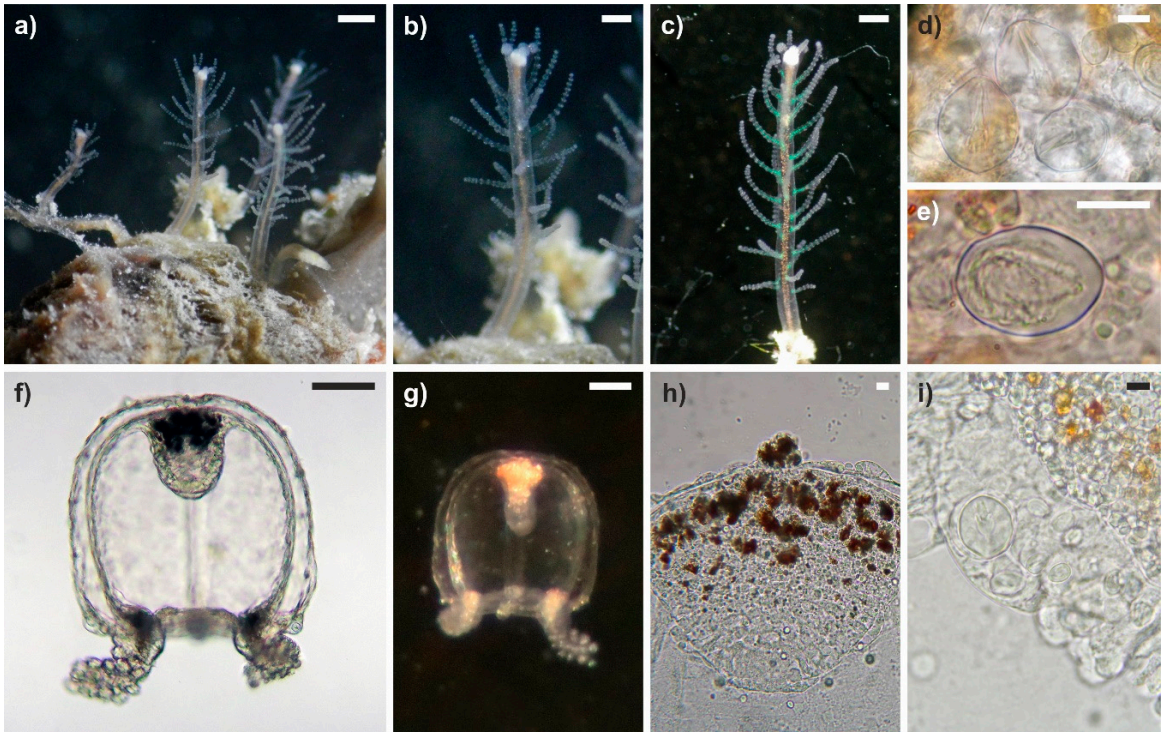

Figure 7. *Asyncoryne ryniensis*. (**a**) Colony growing on dead coral; (**b**) close-up of a polyp; (**c**) polyp showing green fluorescence before stimulation with blue light; (**d**) stenoteles in the capitulum, and (**e**) eurytele in the hydranth; (**f**,**g**) newly released medusa; close-ups of (**h**) manubrium, and (**i**) nematocysts in the tentacular bulb. Scale bars: (**a**) 0.1 mm; (**b**,**c**,**f**,**g**) 0.1 mm; (**d**,**e**,**h**,**i**) 10 µm.

Table 1. Morphology of the polyp stages.

Species	***Zanclea divergens***	***Zanclea* cf. *protecta***	***Zanclea* sp. 1**	***Zanclea* sp. 2**	***Zanclea sango***	***Zanclea* sp. (Clade I)**	***Asyncoryne ryniensis***
Host/Substrate	*Celleporaria vermiformis*	*Parasmittina* cf. *spondylicola*, *Schizoporella* sp.	*Celleporaria pigmentaria*	*Celleporaria* sp.	*Pavona varians*	*Goniastrea* sp.	Rock, sponge
Hydrorhiza	Below the bryozoan skeleton, coming out in irregular notches	On the bryozoan, overgrown by the host skeleton	Below the bryozoan skeleton, coming out in irregular notches	Below the bryozoan skeleton, coming out for some distance	Below the coral skeleton and tissues	Below the coral skeleton and tissues	On the substrate
Perisarc	No	No	No	No	Yes	Yes	Yes
Gastrozooid	Cylindrical, up to 3.5 mm long	Cylindrical, up to 1.5 mm long	Claviform, up to 1.5 mm long	Cylindrical, up to 3 mm long	Cylindrical to claviform, up to 1 mm long	Cylindrical to claviform, up to 1 mm long	Cylindrical, up to 6 mm long
Gastrozooid tentacles	Capitate: 4–5 oral, 16–39 aboral	Capitate: 4–5 oral, 15–44 aboral	Capitate: 4–5 oral, 18–20 aboral	Capitate: 4–5 oral, 23–27 aboral	Capitate: 4–6 oral, 12–22 aboral	Capitate: 4–5 oral, 23–32 aboral	Capitate:3–4 oral; moniliform: 28–36 aboral
Dactylozooid	No	No	No	No	Up to 3 mm, globular apex, no tentacles	Up to 3 mm, globular apex, no tentacles	No
Medusa buds localization	Hydrorhiza	Hydrorhiza and polyps	Hydrorhiza	Hydrorhiza	Hydrorhiza and polyps	Hydrorhiza and polyps	Polyps
Color	Whitish-transparent, white hypostome	Transparent, white hypostome	Transparent, white band, whitish to orange hypostome	Transparent	Transparent, white hypostome	Transparent, white hypostome	Transparent, orange gastroderm, white hypostome
Stenoteles	Capitula	Capitula, hydrorhiza	Capitula, hydrorhiza	Capitula, hydrorhiza	Capitula, hydrorhiza, dactylozooid	Capitula, hydrorhiza, dactylozooid	Capitula, moniliform tentacles, body wall, hydrorhiza

Table 1. *Cont.*

Species	***Zanclea divergens***	***Zanclea* cf. *protecta***	***Zanclea* sp. 1**	***Zanclea* sp. 2**	***Zanclea sango***	***Zanclea* sp. (Clade I)**	***Asyncoryne ryniensis***
Stenoteles size (μm)	11–15 × 9–12, 5–8 × 4–6	11–15 × 8–13, 5–8 × 4–7	14–17 × 12–13, 6–7 × 4–6	18–20 × 12–18, 15–16 × 12–15, 6–7 × 4–6	10–14 × 9–14, 6–8 × 5–6	10–14 × 8–13, 6–9 × 4–6	30–32 × 26–29, 10–11 × 7–8, 8–9 × 6–7
Euryteles	Macrobasic holotrichous, in hypostome, hydrorhiza	No	Macrobasic holotrichous, in hydrorhiza	Macrobasic holotrichous, in hydrorhiza	Macrobasic apotrichous, in hypostome, hydrorhiza, dactylozooid	Macrobasic apotrichous, in hypostome, hydrorhiza, dactylozooid	Macrobasic holotrichous, in body wall, hydrorhiza
Euryteles size (μm)	29–33 × 16–18	No	27–29 × 14–16	18–21 × 11–15	17–21 × 6–10	16–20 × 7–9	20–21 × 15–16

Table 2. Morphology of the newly released medusae.

Species	***Zanclea divergens***	***Zanclea* cf. *protecta***	***Zanclea* sp. 1**	***Zanclea* sp. 2**	***Zanclea sango***	***Zanclea* sp. (Clade I)**	***Asyncoryne ryniensis***
Umbrella	Bell-shaped, diameter: 0.5 mm	Globular to bell-shaped, diameter: 0.5 mm	Globular, diameter: 0.15 mm	Globular, diameter: 0.2 mm	Bell-shaped, diameter: 0.7 mm	Bell-shaped, diameter: 0.5 mm	Bell-shaped, diameter: 0.5 mm
Canals	4 radials, one circular	4 radials, one circular	No	No	4 radials, one circular	4 radials, one circular	4 radials, one circular
Bulbs	4, 2 tentacular larger	4, 2 tentacular larger	2	2	4, 2 tentacular larger	4, 2 tentacular larger	4, 2 tentacular larger
Nematocyst pouches	4, 2 above tentacular bulbs larger	4, same size	No	No	4, same size	4, same size	4, same size

Table 2. *Cont.*

Species	***Zanclea divergens***	***Zanclea* cf. *protecta***	***Zanclea* sp. 1**	***Zanclea* sp. 2**	***Zanclea sango***	***Zanclea* sp. (Clade I)**	***Asyncoryne ryniensis***
Manubrium	Cylindrical, 1/3 of the subumbrellar cavity	Cylindrical, 1/3 of the subumbrellar cavity	Reaching the velar opening, with 4 arms	Protruding from the bell cavity, with 4 arms	Cylindrical, 1/3 of the subumbrellar cavity	Cylindrical, 1/3 of the subumbrellar cavity	Cylindrical, 1/3 of the subumbrellar cavity
Tentacles	2	2	2	2	2	2	2
Cnidophores	21–31, slightly elongated	25–37, elongated	10–15, rounded to elongated	15–17, rounded	35–40, rounded	32–43, rounded	17–24
Color	Transparent, transparent to white manubrium	Transparent, whitish manubrium	Transparent, orange to white manubrium	Transparent, orange to whitish manubrium	Transparent, whitish manubrium	Transparent, whitish manubrium	Transparent, whitish to orange manubrium
Exumbrellar nematocysts (size in μm)	Isorhizae: 5–7 × 5–6	Basitrichous isorhizae: 5–6 × 4–5	Macrobasic holotrichous mastigophores: 7–8 × 6–7	Macrobasic holotrichous mastigophores: 7–9 × 6–8	No	No	Macrobasic holotrichous euryteles: 6–8 × 6–7
Pouches nematocysts (size in μm)	Stenoteles: 12–16 × 11–13	Stenoteles: 11–13 × 9–10	No	No	Macrobasic apotrichous euryteles: 18–19 × 7–9; Stenoteles: 11–12 × 10–11	Macrobasic apotrichous euryteles: 16–20 × 9–11; Stenoteles: 8–10 × 8–9	Stenoteles: 28–29 × 24–26
Manubrium nematocysts (size in μm)	Stenoteles: 5–8 × 4–6	Stenoteles: 6–7 × 5–6	No	No	Stenoteles: 8–10 × 7–8	Stenoteles: 7–8 × 5–6	No
Cnidophores nematocysts (size in μm)	Bean-shaped macrobasic holotrichous euryteles: 7–8 × 5–6	Bean-shaped macrobasic holotrichous euryteles: 7–8 × 5–6	Bean-shaped macrobasic apotrichous euryteles: 5–6 × 4–6	Bean-shaped macrobasic apotrichous euryteles: 6–8 × 4–5	Bean-shaped macrobasic apotrichous euryteles: 7–8 × 4–5	Bean-shaped macrobasic apotrichous euryteles: 7–8 × 4–5	Bean-shaped macrobasic euryteles: 7–8 × 6–7

3.2. Modifications of the Hosts

In all *Zanclea* samples, modification of the skeletons of the hosts were observed. *Zanclea divergens* polyps 'pierced' the skeleton of *Celleporaria vermiformis* along the border between zooids, and in some cases the bryozoan skeleton overgrew the base of polyps as a tube (Figure 1e). The hydrorhiza of *Zanclea* cf. *protecta* growing over the colony of bryozoan host *Parasmittina* cf. *spondylicola* was surrounded by a thin skeletal lamina produced exactly along the border between zooids (Figure 2d). Polyps of *Zanclea* sp. 1 and *Zanclea* sp. 2, associated with *Celleporaria pigmentaria* and *Celleporaria* sp. respectively, were observed coming out from the colony of the hosts at the borders between zooids, being partially overgrown at their base by the skeleton (Figure 3e, Figure 4e). Scleractinian-associated *Zanclea sango* and *Zanclea* sp. caused micro-alterations in the skeleton of the host corals, due to the skeletal overgrowth of the base of polyps and portions of the hydrorhiza (Figures 5f and 6f, respectively).

3.3. Green Fluorescence Essay

The six *Zanclea* and the *Asyncoryne* species showed different patterns of green fluorescence in both the polyp and medusa stages (Figure 8). Specifically, three *Zanclea* species (*Zanclea divergens*, *Zanclea* sp. 1, *Zanclea* sp. 2) and *Asyncoryne ryniensis* did not show fluorescence in the medusa stage. By contrast, the other three *Zanclea* species showed a marked green fluorescence in different structures. *Zanclea* cf. *protecta* showed a fluorescence at the level of the subumbrella, manubrium, and bulbs (Figure 8e,f). *Zanclea* sp. (Clade I) medusae released from colonies associated with *Goniastrea* sp. were characterized by a fluorescence of the radial and circular canals, bulbs, and whole manubrium (Figure 8a,b). Finally, *Zanclea sango* medusae displayed a pattern similar to that of *Zanclea* sp. (Clade I), with the exception of the central portion of the manubrium that did not show any fluorescence (Figure 8c,d). Fluorescence in these medusae was also present when still attached to the parental colony, and showed the same patterns displayed by newly released medusae (Figure 8g,h).

Regarding the polyp stages, *Zanclea* species did not show any fluorescence. Contrarily, *Asyncoryne ryniensis* polyps were characterized by a marked fluorescence at the base of moniliform tentacles (Figure 8i,j). In one polyp, green fluorescence was easily detected without excitation with blue light (Figure 7c).

Fluorescence patterns were identical for all medusae belonging to the same species, and no differences were detected between observations carried out at day one and five after release.

Fluorescence patterns of polyps and medusae for each species are summarized in Table 3.

Table 3. Summary of green fluorescence (GF) patterns in polyps and medusae of *Zanclea* and *Asyncoryne* species.

Species	Host/Substrate	Polyp GF	Medusa GF
Zanclea divergens	*Celleporaria vermiformis*	none	none
Zanclea cf. *protecta*	*Parasmittina* cf. *spondylicola*; *Schizoporella* sp.	none	Subumbrella, manubrium, bulbs
Zanclea sp. 1	*Celleporaria pigmentaria*	none	none
Zanclea sp. 2	*Celleporaria* sp.	none	none
Zanclea sango	*Pavona varians*	none	Manubrium (not in the middle), canals, bulbs
Zanclea sp. (Clade I)	*Goniastrea* sp.	none	Manubrium (whole), canals, bulbs
Asyncoryne ryniensis	Rock, sponge	base of tentacles	none

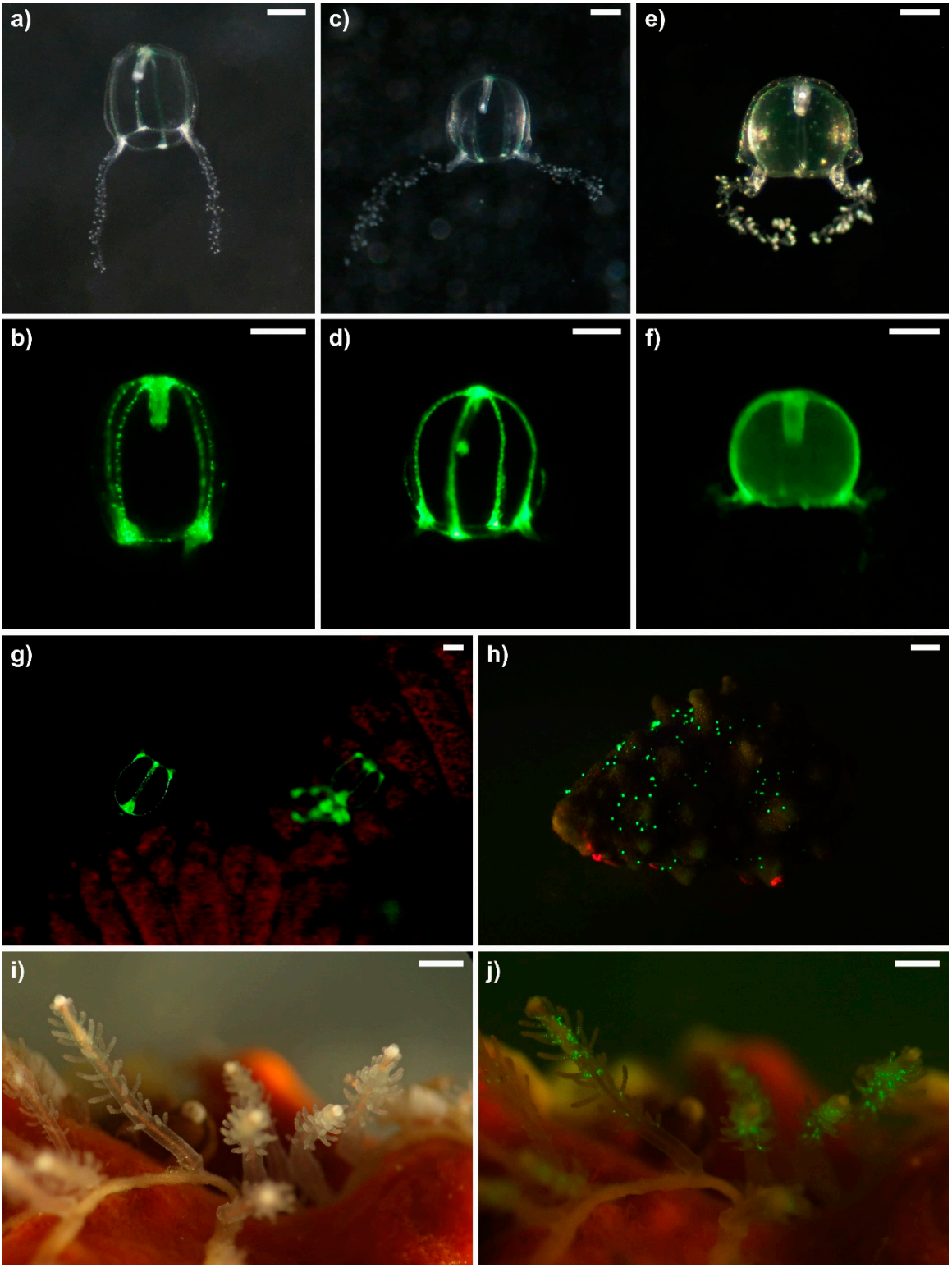

Figure 8. Green fluorescence in *Zanclea* and *Asyncoryne* species. (**a**,**b**) Medusa of *Zanclea* sp. (Clade I) released from a colony associated with *Goniastrea* sp.; (**c**,**d**) medusa of *Zanclea sango;* (**e**,**f**) medusa of *Zanclea protecta*; (**g**) medusa of *Zanclea* sp. before release, associated with *Goniastrea* sp.; (**h**) *Zanclea* cf. *protecta* medusa buds in the colony associated with *Schizoporella* sp. overgrowing the gastropod *Drupella* sp.; (**i**,**j**) *Asyncoryne ryniensis* polyps. Scale bars: (**a**–**g**) 0.2 mm; (**h**) 5 mm; (**i**,**j**) 1 mm.

3.4. 16S rRNA Phylogeny

DNA was extracted successfully, and *16S rRNA* sequences were generated for each analyzed sample. BLASTn searches resulted in a 100% match with previously deposited sequences obtained from Maldivian samples for *Zanclea* sp. 1, *Zanclea* sp. 2, *Zanclea* sp. (Clade I), and *Zanclea sango*. *Zanclea divergens* resulted in a match of 90.7% with an Indonesian sequence of the same species (MF000525), and this low value is explained by the fact that *Z. divergens* is a complex of cryptic species [31]. No *Zanclea protecta* sequences have been deposited so far, and the search for this species resulted in a match of 91.3% with *Zanclea costata* from the Mediterranean Sea (FN687559). Sequences of Maldivian *Asyncoryne ryniensis* resulted in a match of 98.4% with a Japanese specimen (EU876552).

The phylogenetic tree was rooted using *Pennaria disticha* [22,32] and, despite the overall poorly supported relationships (Figure 9), it agrees with previous reconstructions of *Zanclea* phylogeny [22]. Specifically, coral-associated *Zanclea* resulted in a fully supported clade, similarly to the clade composed of *Zanclea* sp. 1 and sp. 2 associated with bryozoans. Moreover, *Z. divergens* was well supported as the sister species of the latter clade, and all three species were associated with *Celleporaria* spp. Finally, the family Zancleidae was confirmed to be polyphyletic, due to the position of *Asyncoryne ryniensis*, which divides the family in two main clades, one associated with corals, and the other with bryozoans.

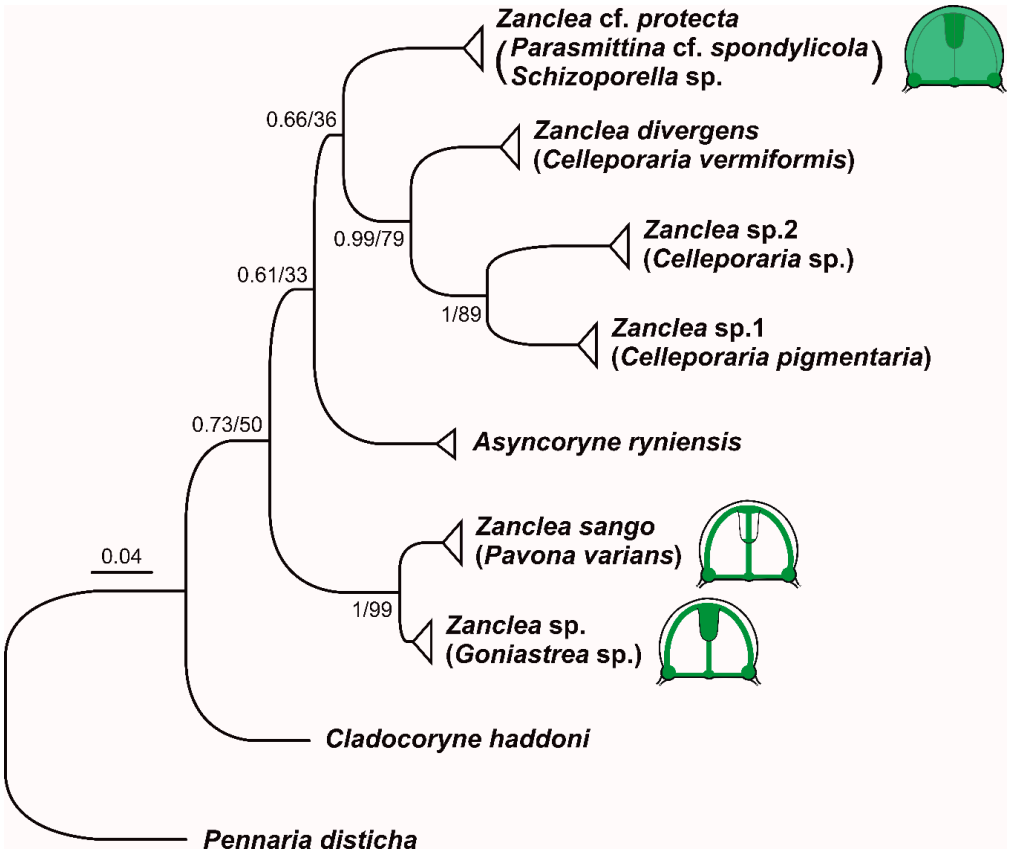

Figure 9. *16S rRNA* phylogeny of the species included in the analyses. Numbers at nodes represent Bayesian posterior probabilities and maximum likelihood bootstrap values, respectively. Hosts for each species are in brackets. Schematic drawings of fluorescence patterns in *Zanclea* medusae are also represented.

Inter-specific genetic distances were high in all comparisons, with the lowest level between the two coral-associated species *Z. sango* and *Zanclea* sp. (Clade I) (4%). All other species showed values higher than 10%. Intra-specific distances were equal to 0% in all cases (Table 4).

Table 4. Pairwise % uncorrected *p*-distances (*16S rRNA*) between all species analyzed.

	(1)	(2)	(3)	(4)	(5)	(6)	(7)
(1) *Z. divergens*	0						
(2) *Z. protecta*	11.7 (1.2)	0					
(3) *Zanclea* sp. 1	10.7 (1.3)	13.2 (1.4)	0				
(4) *Zanclea* sp. 2	12.5 (1.3)	13.7 (1.3)	10.7 (1.2)	0			
(5) *Z. sango*	12.9 (1.3)	13.0 (1.3)	14.9 (1.4)	14.9 (1.4)	0		
(6) *Zanclea* sp. (Clade I)	12.0 (1.3)	12.0 (1.3)	14.0 (1.4)	14.0 (1.3)	4.0 (0.8)	0	
(7) *A. ryniensis*	12.7 (1.3)	11.5 (1.3)	13.2 (1.4)	12.9 (1.3)	13.7 (1.4)	12.3 (1.3)	0

4. Discussion

The genus *Zanclea* and family Zancleidae are challenging taxa both from an evolutionary point of view and for species identification or description [19,22]. Indeed, the genus and family are polyphyletic [22,33], and further analyses are needed to establish new genera or even families. Their taxonomy is complicated by the fact that polyps often have intergrading morphologies, and the adult medusa must be observed and characterized for correct species identification and description [20,22]. Indeed, cryptic or unidentifiable species are common in the *Zanclea* genus [18,19,22].

In this work we analyzed the morphology of six *Zanclea* species, considering the general features of polyps and medusae, the cnidome of both life stages, the alteration of the host skeletal structures, and the green fluorescence patterns. Additionally, we analyzed the molecular identity, phylogenetic relationships and genetic diversity of the species, confirming their possible belonging to six well separated *Zanclea* lineages. Our results show that the characterization of general morphology, and cnidome is in some cases enough to distinguish between *Zanclea* species. For instance, by combining observations on the presence, localization and size of euryteles, and the general appearance of polyps and medusae, it is possible to distinguish the analyzed bryozoan-associated species. By contrast, scleractinian-associated species showed a very similar morphology, as already documented in previous studies [18,21,34].

In all *Zanclea* species here analyzed, alterations of the host skeleton were observed. The bryozoan *Parasimittina* cf. *spondylicola* showed the most evident modification, with the skeletal lamina overgrowing the hydrorhiza of *Zanclea* cf. *protecta*, as already noted by Hasting [35] and Boero et al. [20] for *Zanclea protecta* associated with *Parasmittina crosslandi* (Hastings, 1930) and other unidentified bryozoans. A similar situation was observed for *Celleporaria–Zanclea* associations, where the base of polyps was occasionally surrounded by bryozoan skeletal structures. Additionally, scleractinians hosting *Zanclea* showed micro-alterations related to the presence of symbionts, as already observed in *Goniastrea*, *Pavona*, and *Porites* corals [21]. The presence of these modifications may support the hypothesis that at least some *Zanclea* species are mutualistically associated with their hosts, since they may provide additional protection and competitive advantages to their hosts and in turn benefit from being partially enclosed in hard carbonatic structures [36,37].

Differences were found in the green fluorescence patterns of *Zanclea* and *Asyncoryne* species. *Zanclea divergens*, *Zanclea* sp. 1 and *Zanclea* sp. 2 did not show any fluorescence neither in the polyps nor in the medusae. *Zanclea* cf. *protecta*, *Zanclea sango*, and *Zanclea* sp. (Clade I) did not show any fluorescence in the polyps but medusae were characterized by different green fluorescence patterns. Finally, *Asyncoryne ryniensis*, which has different polyps but medusae very similar to those of *Zanclea*, showed fluorescence in polyps but not in medusae. *Zanclea* cf. *protecta* is characterized by a diffuse fluorescence in bulbs, manubrium, and subumbrella, whereas *Z. sango* and *Zanclea* sp. (Clade I) are fluorescent in bulbs, manubrium, and canals. Despite the two latter coral-associated species have overlapping morphologies in both polyp and medusa stages, medusae showed differences in the distribution of green fluorescent proteins at the level of manubrium. Specifically, in *Zanclea* sp. (Clade I) the entire manubrium is fluorescent, and this pattern is visible even in the medusa buds still attached to the parental colony, whereas in *Zanclea sango* fluorescence is concentrated at the extremes

of manubrium (mouth and close to the umbrellar margin), being absent in the middle portion. These conditions were observed in all analyzed medusae and therefore may be taxonomically informative, even if further analyses are needed to confirm this hypothesis. Fontana et al. [38] also found green fluorescence in medusa buds of *Acropora*-associated *Zanclea* species, but the localization was not reported. However, this suggests that, potentially, the medusae of other coral-associated *Zanclea* species may be fluorescent. If this is true, the investigation of fluorescence patterns in all *Zanclea* species associated with scleractinians may help disentangling the cryptic diversity that characterize this group.

The function, if any, of green fluorescent proteins in the analyzed species is still not clear. One of the possible explanations is attraction of prey [8]. The polyps of the six *Zanclea* species observed all live in symbiosis with other organisms, and the lack of fluorescence in this stage may be related to specific feeding interactions with the hosts, as described for *Zanclea divergens*, which seems to feed on mucous aggregates of particles egested by the bryozoan [39]. Moreover, *Asyncoryne ryniensis* is not symbiotic, and fluorescence is found at the base of polyp tentacles. This explanation complicates with the medusa stages, since species with potentially similar feeding behaviors show contrasting fluorescence patterns.

Overall, the results obtained in this work show that the combination of multiple approaches allows one to discriminate closely related *Zanclea* species and provide information on the relationships between these hydrozoans and their hosts. Additionally, the analysis of green fluorescence patterns seems to be a promising tool for hydrozoan taxonomy and should be performed at a large scale to assess its adequacy in exploring and distinguishing the diversity of enigmatic hydrozoan taxa, such as zancleids.

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