Acta Oceanol. Sin., 2022, Vol. 41, No. 3, P. 44–52
https://doi.org/10.1007/s13131-021-1900-5
http://www.aosocean.com
E-mail: ocean2@hyxb.org.cn

Discovery of *Cladonema multiramosum* sp. nov. (Cnidaria: Hydrozoa: Cladonematidae) using DNA barcoding and life cycle analyses

Konglin Zhou[1]*, Yuping Gu[1], Lu Wang[1], Jianming Chen[1]*

[1] Institute of Oceanography, College of Geography and Oceanography, Minjiang University, Fuzhou 350108, China

Received 25 July 2021; accepted 13 September 2021

Abstract

In contrast to typical planktonic hydromedusae, *Cladonema* medusae are mostly benthic, with specialised adhesive branches to adhere to the substrate. In this study, a *Cladonema* species discovered in a laboratory aquarium in Fuzhou, China, was confirmed as a new species, based on morphological and molecular analyses. The species was named *Cladonema multiramosum* sp. nov. Its medusa is distinct from that of congeners possessing substantially more adhesive branches (8–24, rarely 5–7), and tiny branches on the upper radial canals. The validity of *C. multiramosumum* sp. nov. was also supported by molecular phylogenetic analyses based on the mitochondrial 16S rDNA sequence. However, *C. multiramosum* sp. nov. medusa also displayed considerable phenotypic plasticity with respect to its radial canals, tentacles, stinging branches per tentacle, oral tentacles, manubrium, and gonads, hindering species identification based solely on morphology. Although some morphological characteristics of hydroids (filiform tentacles and medusa buds) and nematocysts could also be used as diagnostic characters in the genus *Cladonema*, this information is unavailable for some *Cladonema* species. Thus, the taxonomy within the genus *Cladonema* was re-evaluated based mainly on the morphological characteristics of the medusa. Further revision of the genus *Cladonema* should be made when supplementary information on the life cycle and DNA barcoding are updated.

Key words: *Cladonema*, morphology, life cycle, DNA barcoding, 16S rDNA

Citation: Zhou Konglin, Gu Yuping, Wang Lu, Chen Jianming. 2022. Discovery of *Cladonema multiramosum* sp. nov. (Cnidaria: Hydrozoa: Cladonematidae) using DNA barcoding and life cycle analyses. Acta Oceanologica Sinica, 41(3): 44–52, doi: 10.1007/s13131-021-1900-5

1 Introduction

Cladonema species are widely distributed in the coastal waters of the Atlantic Ocean, Indian Ocean, Mediterranean Sea, Black Sea, Bermuda, Brazil, New Zealand, Australia, Japan, and China (Zhou and Huang, 1958; Schuchert, 2006; Gershwin and Zeidler, 2008; Cedeño-Posso, 2014; Xu, 1993). Unlike the pelagic medusa stage of most hydromedusae, *Cladonema* medusae typically adhere to substrates using adhesive tentacles (Schuchert, 2006). Therefore, they are not frequently caught in plankton nets, leading to their absence in conventional zooplankton samples. Consequently, the distribution ranges and abundances of *Cladonema* species are most likely underestimated. In recent decades, increasing jellyfish blooms have resulted in adverse ecological effects in marine habitats (Purcell, 2018). Abundant *Cladonema radiatum* appeared from June to August in Yantai waters, China; they may act as an important predator in local habitats (Zhou and Huang, 1958). Additionally, *C. pacificum* and *C. radiatum* have been chosen as model organisms to study biological development and regeneration, such as cell proliferation, branching morphogenesis, and eye development (Graziussi et al., 2012; Fujiki et al., 2019; Fujita et al., 2019).

To date, six valid species have been accepted in the genus *Cladonema* (Schuchert, 2021), but the phenotypic plasticity of their medusae has led to several rounds of lumping and splitting among *Cladonema* species, especially for *C. radiatum* and its subspecies (Schuchert, 2006; Gershwin and Zeidler, 2008). An additional obstacle for species distinguishability is the lack of descriptions of the polyp stage of some species, such as *C. novaezelandiae*, *C. timmsii*, and some subspecies (Schuchert, 2006; Gershwin and Zeidler, 2008). Although life-cycle observations of the hydromedusae could be essential for their identification (Bouillon et al., 2006), cultivation experiments are difficult and time consuming (Schuchert et al., 2017). Generally, it is not easy or impossible to identify species of immature *Cladonema* medusae. In Cnidaria, DNA barcoding based on mitochondrial cytochrome oxidase (COI) and mitochondrial 16S rDNA (16S) has been proven to be efficient and reliable for identifying hydromedusae (Bucklin et al., 2010a, 2010b; Zheng et al., 2014; Schuchert, 2016), and detecting cryptic or new species (Lindner et al., 2011). Thus, morphological descriptions, life-cycle observations, and molecular phylogenetic analyses should be combined to accurately describe new/cryptic hydromedusae (Zhou et al., 2013; He et al., 2015).

In this study, a *Cladonema* species was discovered in the

Foundation item: The Fund of China Ocean Mineral Resources R&D Association under contract No. DY135-B2-13; the National Natural Science Foundation of China under contract No. 41806181; the Natural Science Foundation of Fujian Province, China under contract No. 2021J011042; the Program for Innovative Research Team in Science & Technology in Fujian Province University; the Talent Introduction Pre-research Project of Minjiang University under contract No. MJY20017.
*Corresponding author, E-mail: zhoukl@ mju.edu.cn; chenjm@mju.edu.cn

Oryzias melastigma aquariums in our laboratory (Fuzhou, China), which was turned out to be a new species after morphological and molecular examination. We further re-evaluated the taxonomy of *Cladonema* species.

2 Materials and methods

2.1 *Sample collection*

The *Cladonema* species was found in the *Oryzias melastigma* aquariums in our laboratory. The fish were obtained from the City University of Hong Kong and were fed with *Artemia* sp. nauplii from Tibet, China. Polyps of the *Cladonema* species were collected from the experimental aquaria and kept alive in artificial seawater prepared using sea salt (Haike, Qingdao, China) and pure water for morphological and life cycle observations. Individuals used for molecular examination were acclimated in artificial seawater for at least 48 h.

2.2 *Culturing and morphological description*

Hydroids were reared in covered glass vessels (diameter: 180 mm) with a polyethylene plate placed at the bottom to allow the hydroids to attach to it. The culture temperature was maintained at approximately 25°C using an air conditioner; salinity was tested each day and maintained between 28 and 30. The polyps were fed with *Artemia* sp. nauplii every second day, and the seawater was changed after feeding to keep the culture environment clean. Newly released medusae were collected and reared to maturity under the same culture conditions as those used for morphological analyses. Both hydroids and medusae were observed under a stereomicroscope (Leica E24W, Leica Microsystems, Germany) before feeding to record their development.

Thirty hydroids, newly released medusae, and mature medusae each were picked randomly, photographed, and measured using a stereomicroscope (Leica M165FC, Leica Microsystems, Germany). The hydroids and medusae were narcotized by adding 10% $MgCl_2$ solution to the seawater before observation. Several hydroids and medusae were selected to determine their nematocyst types and distribution using a light microscope equipped with differential interference contrast optics (Leica SP8, Leica Microsystems, Germany). The nematocyst nomenclature followed that of Östman (2000). Distilled water was used to induce the discharge of nematocysts during squash preparation (Östman, 1987).

2.3 *Molecular analyses*

The DNA was extracted from pools of five hydroids using a DNeasy Blood & Tissue Kit (Qiagen, QIAGEN GmbH, Germany) according to the manufacturer's instructions, with five replicates. The morphology of the polyps was checked under a stereoscopic microscope, after which they were carefully cleaned with artificial seawater before DNA extraction. Partial mitochondrial 16S (~0.6 kb) and mitochondrial COI (~0.7 kb) fragments were amplified using 2×Ex Taq Mastermix (Takara Bio, Japan) and the primers listed in Table 1. Reaction mixtures (50 μL) were prepared according to the manufacturer's instructions. The polymerase chain reaction (PCR) programs for 16S were as follows: 94°C for 1 min, followed by 35 cycles at 94°C for 50 s, 50°C for 60 s, and 72°C for 60 s, and a final extension at 72°C for 5 min. The PCR program for COI was as follows: 94°C for 4 min, followed by 35 cycles at 94°C for 40 s, 50°C for 60 s, and 72°C for 90 s, and a final extension at 72°C for 10 min. The PCR products were purified using a Multifunction DNA Purification Kit (Biomed, China) and then cloned into a pMD-19T vector (Takara Bio, Japan). The pMD-19T constructs were transformed into *Escherichia coli* JM109 competent cells (Takara Bio, Japan). Monoclonal colonies containing target DNA fragments were selected by PCR and then sent to Sangon Biotech Co., Ltd (China) for sequencing using an ABI 3730 automatic DNA sequencer with the universal M13 primer. All samples were sequenced in both directions to ensure sequence accuracy.

Sequences were examined based on the chromatogram files. Target sequences were captured with the primers of COI and 16S, aligned using the NCBI Nucleotide Blast program to confirm their accuracy and validity, and deposited in GenBank (Table 1). Pairwise genetic distances of COI and 16S fragments in Cladonematidae species were calculated using MEGA-X with the Kimura-2-Parameter (K2P) model. Mean comparisons were conducted using the SPSS16.0. The normality and homogeneity of data were tested. One-way analysis of variance (ANOVA) and *t*-test were used to assess differences in genetic distances among different levels.

Since only three COI sequences of Cladonematidae species were found in GenBank, only the 16S sequences of Cladonematidae species in GenBank were used to infer the phylogenetic trees using maximum-likelihood (ML, based on the GTR+G+I model) in PhyML 3.0 (Guindon et al., 2010) and neighbour-joining (NJ, based on the K2P model) in MEGA-X (Tamura et al., 2011) with 1 000 bootstrap replicates. Corynidae medusae *Coryne eximia* (AY512541 and AJ878713), *Coryne pusilla* (AY512552 and AY787874) and *Sarsia tubulosa* (GQ395327 and EU876548) were chosen as outgroups (Nawrocki et al., 2010).

3 Results

Systematics

Phylum Cnidaria Hatschek, 1888
Class Hydrozoa Owen, 1843
Subclass Hydroidolina Collins, 2000
Order Anthoathecata Cornelius, 1992
Suborder Capitata Kühn, 1913
Family Cladonematidae Gegenbaur, 1857
Genus *Cladonema* Dujardin, 1843
Cladonema multiramosum sp. nov.
(Figs 1–3; Tables 2 and 3)

Material examined

Holotype: MJU-HYD-1, male medusa, diameter 1.85 mm, height 1.65 mm.

Paratypes: MJU-HYD-2, a lot of 30 mature medusae (21 males, 9 females), diameter 1.57–2.26 mm, height 1.36–1.95 mm. MJU-HYD-3, a large number of 30 newly released medusae, with diameter of 0.60–0.92 mm and height of 0.40–0.69 mm. MJU-

Table 1. PCR primers and GenBank accession numbers of sequences used in this study

Gene	Primer name	Sequence (5' to 3')	GenBank accession number	Reference
16S	16S-L	GACTGTTTACCAAAAACATA	MW714350–MW714354	Ender and Schierwater (2003)
	16S-H	CATAATTCAACATCGAGG		
COI	LCO-1490	GGTCAACAAATCATAAAGATATTGG	MW714344–MW714348	Folmer et al. (1994)
	HCO-2198	TAAACTTCAGGGTGACCAAAAAATCA		

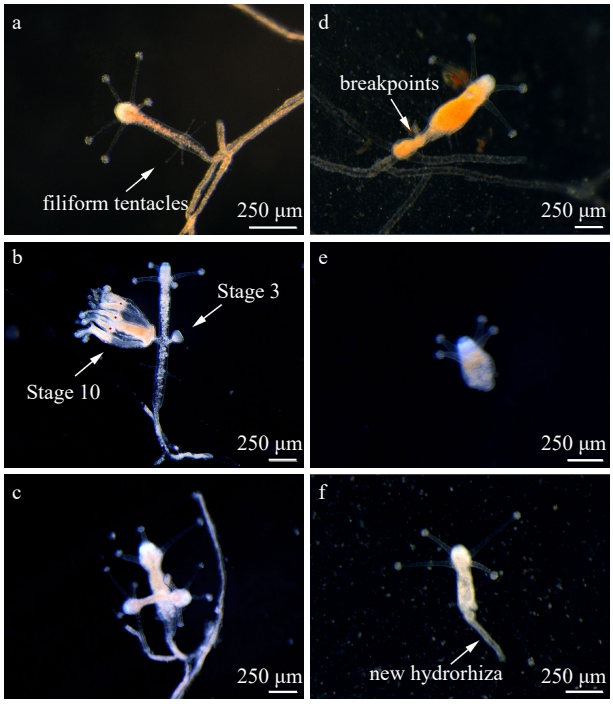

Fig. 1. Hydroids of *Cladonema multiramosum* sp. nov. a. Hydranth; b. polyp with medusa buds; c. polyp with hydranth buds; d. hydranth before breaking; e. released hydranth; f. re-attached hydranth.

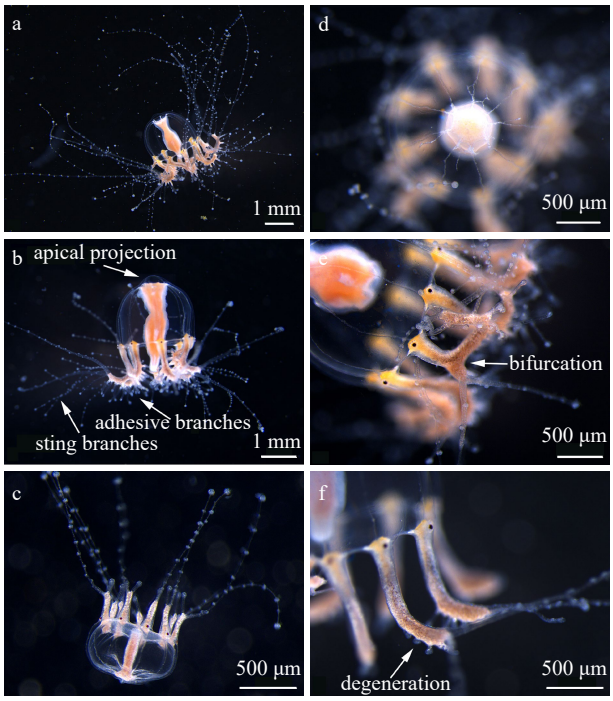

Fig. 2. Medusa of *Cladonema multiramosum* sp. nov. a. Mature male medusa; b. mature female medusa; c. newly released medusa; d. planiform of medusa; e. the branches of marginal bulbs; f. degenerated tentacle.

HYD-4, polyps with both trophosomes and immature medusa buds.

All specimens were obtained from individuals cultured in the laboratory in February 2020 and deposited at the Institute of Oceanography, Minjiang University, Fuzhou, China.

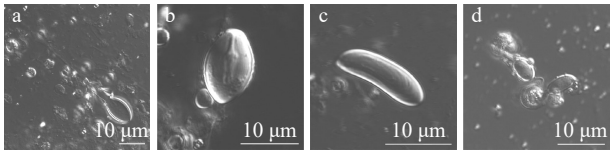

Fig. 3. Interference contrast micrographs of the nematocyst from *Cladonema multiramosum* sp. nov. a and b. Stenoteles; c. mastigophores; d. desmonemes.

Table 2. Measurements (mean±SD (range)) of polyps of *Cladonema multiramosum* sp. nov. (*n*=30)

Parts	Parameters	Results
Hydranth	height/mm	0.61±0.18 (0.30–0.98)
	width/mm	0.08±0.02 (0.05–0.12)
Oral tentacle	type	capitate
	number	4–6
	length/mm	0.29±0.09 (0.15–0.45)
Aboral tentacles	type	filiform
	number	4–6
Medusa buds	position	between oral and aboral tentacles
	number	1–5
Hydrocaulus	length/mm	0.10±0.06 (0.03–0.22)
	width/mm	0.03±0.01 (0.02–0.06)
Hydrorhiza	width/mm	0.03±0.01 (0.02–0.06)

Table 3. Measurements (mean±SD (range)) of medusae of *Cladonema multiramosum* sp. nov. (*n*=30)

Parts	Parameters	New released medusa	Mature medusa
Umbrella	height/mm	0.52±0.06 (0.40–0.69)	1.60±0.18 (1.36–1.95)
	diameter/mm	0.74±0.08 (0.60–0.92)	1.91±0.19 (1.57–2.26)
Apical projection	height/mm	absent	0.25±0.06 (0.13–0.39)
Radial canals	number	6–11	8–11
	type	simple straight	branched
Marginal tentacles	number	6–11	8–11
	the number of adhesive branches	1–3, mainly 1	5–24
	the number of stinging branches	1	2–6
Ocelli	location	on bulbs	on bulbs
	number	6–11	8–12
	colour	red–brown	black
Manubrium	length/mm	0.46±0.05 (0.34–0.55)	1.50±0.34 (0.78–2.51)
	width/mm	0.13±0.02 (0.10–0.16)	0.64±0.16 (0.44–0.99)
Oral tentacle	number	absent	5–8
Gonad	length/mm	absent	1.23±0.29 (0.62–2.03)
	colour	absent	white

Diagnosis

Polyp: Hydranth with one oral whorl of generally 4–6 capitate tentacles and one aboral whorl of generally 4–6 slender, filiform tentacles. Medusa buds naked, up to five per polyp, born singly on the hydranth body between capitate and filiform tentacles. Nematocysts:

(a) stenoteles, (10.2–18.6) μm × (6.5–10.9) μm.

(b) mastigophores, rare or absent, in stolons only, (11.1–14.3) μm ×

(3.3–4.7) μm.

Adult medusa: umbrella bell-shaped with an apical projection on the top, medusa 1.36–1.95 mm high and 1.57–2.26 mm wide, jelly moderately thin, velum broad. Manubrium with 5–8 pouches (usually six) and able to extend beyond the umbrella margin, with gonads completely encircling it in the upper 4/5. Mouth with 5–8 bulbous nematocyst clusters, mostly six. Radial canals 8–11, straight, issuing directly from the stomach to the circular canal, some with several tiny branches. Tentacles 8–11, each bearing 3–6 stinging branches (rarely two) and 8–24 adhesive branches (rarely 5–7). The tentacle base was bent cylindrical, with a black abaxial ocellus, rarely two. Tentacle bases elongated and thickened as the number of adhesive branches increased. Nematocysts:

(a) stenoteles, (7.5–18.5) μm × (4.6–11.9) μm.

(b) desmonemes, (6.2–9.0) μm × (3.3–5.1) μm.

Description

Hydroid: The morphological characteristics of the hydroids are presented in Fig. 1 and Table 2. Hydroids stolonal, rarely branched, and arising from ramified stolon with a short hydrocaulus. The hydranth naked, variable in size: 0.30–0.98 mm high and 0.05–0.12 mm wide. The hydrocaulus and stolon were covered with a smooth perisarc. Hydranth with one oral whorl of 4–6 capitate tentacles, rarely 10, 0.15–0.45 mm long under anaesthesia and ending with bulbous nematocyst clusters. One aboral whorl of 4–6, rarely more than 10, slender, filiform tentacles located about 4/7–6/7 of the distance from the hypostome to the base. Medusae buds naked, not covered by a visible periderm, up to five per hydranth, at different developmental stages, located between capitate and filiform tentacles, generally close to filiform tentacles (Fig. 1b). In addition, a few hydranths had hydropolyps instead of medusa buds (Fig. 1c).

Newly released medusa: The morphological characteristics of the medusa stage are shown in Fig. 2 and Table 3. Newly-released medusa with bell-shaped umbrella, not completely stretched, 0.60–0.92 mm wide and 0.40–0.69 high. Exumbrella surface smooth, jelly thin, velum broad. Manubrium spindle-shaped, 0.34–0.55 mm long, shorter than or as long as bell cavity. Radial canals 8–11, rarely 6–7, fine, straight, reaching the circular canal, rarely branched. Tentacle bulbs 8–11, rarely 6–7, one opposite each radial canal, hourglass-shaped, with an abaxial red-brown ocellus on a tentacle base. One stinging tentacle branch and 1–3 (mainly one) adhesive branch per tentacle bulb.

Adult medusa: Adult medusa with bell-shaped umbrella, 1.57–2.26 mm wide and 1.36–1.95 mm high, exumbrella surface smooth, jelly moderately thin with an apical projection (Fig. 2b), velum broad. Manubrium columnar or spindle-shaped, variable in length (0.78–2.51 mm), able to extend beyond the umbrella margin, with 5–8 pouches (usually six), with opalescent gonads completely encircling it in the upper 1/2–1 (mean 4/5). Female gonad grainy; male, smooth. Mouth with 5–8 bulbous nematocyst clusters, mostly six. Radial canals 8–11, most straight, issuing directly from the stomach to the circular canal some radial canals bifurcate close to the manubrium, and some with several tiny branches on the top. Tentacle bases 8–11, one opposite each radial canal, bent cylindrical. One ocellus per bulb (rarely two), black, positioned abaxially at the top of the tentacle base. Tentacles 8–11, emitting from each base, each bearing 3–6 stinging branches (rarely two), and 8–24 adhesive branches (rarely 5–7). Tentacle bases extended with an increase in the adhesive tentacles. Some tentacle bases bifurcate, emitting two tentacles (Fig. 2e). Orange stomach and tentacle base. Medusa can swim freely but remains attached using adhesive tentacles.

Distribution

Hitherto, this species was only found in the *O. melastigma* aquaria in our laboratory, Fuzhou, China. *Cladonema multiramosum* may originate from Hong Kong through the introduction of *O. melastigma* from City University of Hong Kong, or from a salt lake in Tibet along with *Artemi*a sp. nauplii. Further field distribution and seasonal variations remain unresolved.

Etymology

This species is named after the Latin term *multiramosum* because it has many adhesive branches (8–24), more than that of other species in the genus *Cladonema*.

Biological notes

When the temperature was maintained at 15–30°C and salinity at 26–33, and animals were fed every 1–3 d, hydroids could be easily reared. Higher and lower temperatures reduced the number of colonies. Sometimes, the hydranth fractured and separated itself from the stolon or hydrocaulus. The hydranth part reattached to a new substrate as soon as possible (Figs 1d–f). Abundant medusa buds were observed at 20–25°C. Newly released medusae were attracted by light, but later lost this preference and adhered to the bottom after one day of development. The medusae matured after 20 d at 25°C. During the developmental period, both the stinging and adhesive branches increased with the elongation of the tentacle bases, and the short branches in the radial canals also increased. Adhesive branches reduced with aging of the medusae (Fig. 2f).

DNA barcoding

For both the 16S (558 bp) and COI (709 bp) sequences, genetic divergences were 0–0.005 and 0–0.003, respectively, among individuals of *C. multiramosum* sp. nov. For 16S, the pairwise intra-species K2P genetic distances (0–0.051) did not overlap with intra-genus distances within the genera *Cladonema* (0.091–0.300), *Staurocladia* (0.113–0.211), and *Eleutheria* (0.217–0.230), as shown in Table 4, indicating an obvious "barcoding gap". *Cladonema multiramosum* sp. nov. displayed the least genetic divergence with *Cladonema* sp. [MT709261: 0.092±0.001 (0.091–0.093)]; the second closest species was *C. radiatum* [0.111±0.003 (0.106–0.117)], and the third closest species was *C. pacificum* [0.140±0.004 (0.136–0.145)]. *Cladonema* sp. (AM088484) showed large genetic divergences [0.296±0.003 (0.294–0.300)] with *C. multiramosum* sp. nov., which were similar to the divergences with *Cladonema*, *Staurocladia, Eleutheria* and even Corynidae medusa [0.291±0.009 (0.282–0.302)] in outgroups (Table 4; ANOVA, F=1.944, p=0.144). Without *Cladonema* sp. (AM088484), the intra-species distances [0.018±0.020 (0–0.051)] were smaller than intra-genus distances [0.140±0.038 (0.091–0.230), t-test, t=–18.839, p<0.01], which were also smaller than intra-family distances [0.190±0.040 (0.094–0.257), t-test, t=–10.335, p<0.01] in Cladonematidae. The genetic distances between *Cladonema* sp. (AM088484) and other Cladonematidae medusa were 0.276±0.022 (0.239–0.327), which were larger than the intra-family distances (t-test, t=11.274, p<0.01). For COI, *C. multiramosum* sp. nov. showed similar genetic distances with *Staurocladia wellingtoni* (MF000486, 0.171) and *Staurocladia vallentini* (MF000500, 0.177–0.178), and larger genetic distances with *C. radiatum* (MF000495, 0.220–0.224), within the range of intra-family distances based on 16S.

The NJ and ML topologies of Cladonematidae based on 16S revealed an independent clade of *C. multiramosum* sp. nov. with strong support in the genus *Cladonema* (Fig. 4). We recovered that the genera *Cladonema*, *Eleutheria* and *Staurocladia* are polyphyletic, and *Eleutheria dichotoma* is nested within the *Staurocladia* clade.

Table 4. Pairwise Kimura 2 Parameter (K2P) genetic distances (mean±SD (range)) between species in family Cladonematidae based on mitochondrial 16S rDNA

	Staurocladia wellingtoni	*Staurocladia vallentini*	*Staurocladia oahuensis*	*Staurocladia bilateralis*	*Staurocladia* sp. (MT709274)	*Eleutheria claparedii*
Staurocladia wellingtoni	**0**					
Staurocladia vallentini	0.188±0.015 (0.169–0.205)	**0.038**				
Staurocladia oahuensis	0.197±0.003 (0.195–0.201)	0.118	–			
Staurocladia bilateralis	0.208±0.003 (0.205–0.211)	0.121±0.012 (0.113–0.129)	0.157	–		
Staurocladia sp. (MT709274)	0.179±0.007 (0.172–0.186)	0.121±0.009 (0.115–0.128)	0.156	0.133	–	
Eleutheria claparedii	0.185±0.003 (0.183–0.189)	0.213±0.011 (0.205–0.221)	0.202 673 646	0.217 316 434	0.226	–
Eleutheria dichotoma	0.196±0.008 (0.184–0.209)	0.110±0.012 (0.094–0.128)	0.132±0.005 (0.126–0.138)	0.105±0.007 (0.097–0.113)	0	0.223±0.006 (0.217–0.230)
Cladonema pacificum	0.156±0.002 (0.155–0.160)	0.219±0.019 (0.196–0.242)	0.236±0.007 (0.231–0.241)	0.255±0.004 (0.252–0.257)	0.228±0.007 (0.223–0.232)	0.197±0.002 (0.195–0.198)
Cladonema radiatum	0.127±0.008 (0.116–0.138)	0.198±0.013 (0.181–0.221)	0.190±0.011 (0.182–0.207)	0.216±0.007 (0.208–0.226)	0.199±0.010 (0.190–0.210)	0.192±0.010 (0.182–0.208)
Cladonema sp. (AM088484)	0.242±0.003 (0.239–0.245)	0.258	0.283	0.237	0.274	0.241
Cladonema sp. (MT709261)	0.121±0.003 (0.117–0.123)	0.170±0.012 (0.162–0.178)	0.206	0.203	0.179	0.195
Cladonema multiramosum sp. nov.	0.186±0.003 (0.182–0.193)	0.195±0.006 (0.188–0.204)	0.215±0.001 (0.214–0.217)	0.219±0.002 (0.218–0.223)	0.233±0.002 (0.232–0.237)	0.231±0.001 (0.231–0.233)

	Eleutheria dichotoma	*Cladonema pacificum*	*Cladonema radiatum*	*Cladonema* sp. (AM088484)	*Cladonema* sp. (MT709261)	*Cladonema multiramosum* sp. nov.
Staurocladia wellingtoni						
Staurocladia vallentini						
Staurocladia oahuensis						
Staurocladia bilateralis						
Staurocladia sp. (MT709274)						
Eleutheria claparedii						
Eleutheria dichotoma	**0.030±0.022 (0.002–0.051)**					
Cladonema pacificum	0.241±0.007 (0.232–0.247)	**0.002**				
Cladonema radiatum	0.200±0.011 (0.186–0.221)	0.128±0.006 (0.121–0.137)	**0.021±0.016 (0–0.036)**			
Cladonema sp. (AM088484)	0.291±0.002 (0.289–0.294)	0.255±0.012 (0.247–0.264)	0.274±0.013 (0.261–0.295)	–		
Cladonema sp. (MT709261)	0.193±0.007 (0.186–0.201)	0.137±0.010 (0.131–0.144)	0.111±0.007 (0.105–0.121)	0.262	–	
Cladonema multiramosum sp. nov.	0.227±0.015 (0.210–0.244)	0.140±0.004 (0.136–0.145)	0.111±0.003 (0.106–0.117)	0.296±0.003 (0.294–0.300)	0.092±0.001 (0.091–0.093)	**0.002±0.002 (0–0.005)**

Note: The pairwise intra-species K2P genetic distances were in bold. – represents absent.

4 Discussion

The medusa of *C. multiramosum* sp. nov. is distinguishable from other nominal *Cladonema* species in having many adhesive branches (8–24, rarely 5–7), and tiny irregular side-branches on the upper radial canals. The validity of *C. multiramosum* sp. nov. has been confirmed by both morphological and molecular analyses.

4.1 *Morphological differences among Cladonema species*

A comparison of *C. multiramosum* sp. nov. with other nominal *Cladonema* species is displayed in Tables 5–7. In the hydroid stage, *C. multiramosum* sp. nov. differs from *C. pacificum* and *C. myersi*, which lack filiform tentacles (Rees, 1950; Hirai, 1958; Takeda et al., 2018), whereas it is similar to *C. californicum* and *C. radiatum*, which possess an aboral whorl of filiform tentacles (Rees, 1979; Schuchert, 2006). However, *C. multiramosum* sp. nov. has more medusa buds per polyp (1–5) than *C. californicum* (2–3) and *C. radiatum* (1) (Rees, 1979; Schuchert, 2006). In addition, the medusa buds of *C. multiramosum* sp. nov. were scattered between the capitate and filiform tentacles, whereas those of *C. californicum* were arranged in a single whorl (Rees, 1979). Thus, the existence of the filiform tentacles of hydroids, and the number and arrangement of medusa buds on the hydroids can be used as diagnostic characters in *Cladonema* polyps. However, the polyps of *C. multiramosum* sp. nov. could not be distinguished from that of *C. novaezelandiae* and *C. timmsii* because the polyps of these two nominal species are unknown (Ralph, 1953; Gershwin and Zeidler, 2008).

In the mature medusa stage, the validity of *C. multiramosum* sp. nov. as a new species could be supported by distinct identifiable morphological characteristics, such as the increased number of adhesive branches (8–24, rarely 5–7), and tiny branches on the upper radial canals, compared with other *Cladonema* medusae (Table 6). Very few mature medusae of *C. multiramosum* sp.

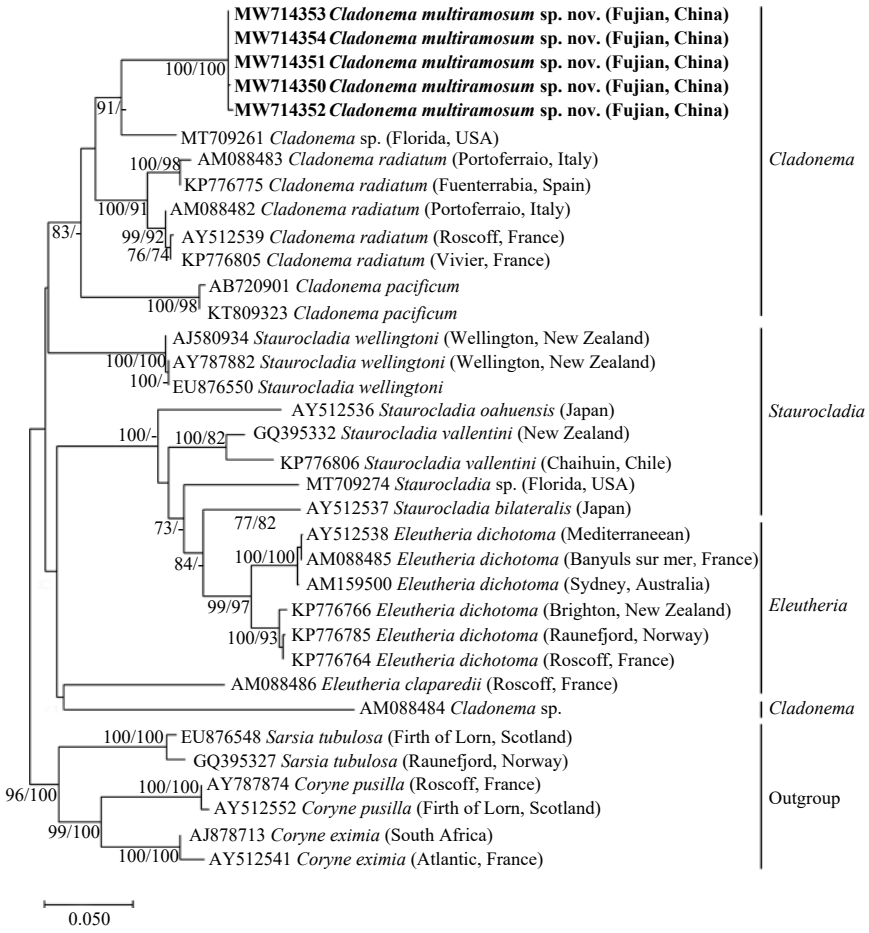

Fig. 4. Neighbour-joining clustering of Cladonematidae based on mitochondrial 16S rDNA. Bootstrap values of 1 000 pseudoreplicates higher than 70% were shown above the branches as node-support values. The first number along the branches refers to the neighbour-joining bootstrap values, while the second number refers to the maximum likelihood bootstrap values.

nov. shared a similar number of adhesive branches (5–7) with *C. novaezelandiae* (up to seven) and *C. timmsii* (5–7) (Ralph, 1953; Gershwin and Zeidler, 2008). Although some *C. radiatum* medusae have a slight apical projection, they lack tiny branches on the radial canals and have fewer adhesive branches (1–4) than *C. multiramosum* sp. nov. (Schuchert, 2006; Cedeño-Posso, 2014; Farias et al., 2020; Ghory et al., 2020).

In addition to these distinctive features, the medusae of *C. multiramosum* sp. nov. showed variability in the numbers of radial canals, tentacles, stinging branches per tentacle, pouches in the manubrium, and oral tentacles, as well as the relative positions of the manubrium and gonad (Table 6), which are generally used in the classification of *Cladonema* medusa (Schuchert, 2006). All these observed morphological differences of the nominal *Cladonema* species are very variable, of low complexity and thus difficult to use to distinguish species. Moreover, the differences between the nominal species could, in many cases, simply be due to stochastic effects because only very few specimens have been examined, and these were often of clonal origin.

The radial canals of *C. multiramosum* sp. nov. are straight, while some bifurcate near their origin, which also occurred in other *Cladonema* medusae (Table 6). The number of radial canals in *C. multiramosum* sp. nov. is variable, covering the range seen in other *Cladonema* medusae except for *C. myersi* (seven, rarely 5–6) (Rees, 1950, 1982), as well as the medusa tentacles. Sometimes, it was difficult to distinguish *C. mul-*

Table 5. Comparison of the morphology of polyps in the genus *Cladonema*

Species	Capitate tentacles	Filiform tentacles	Medusae buds	Reference
Cladonema californicum	4	4–5	2–3, in a single whorl between capitate and filiform tentacles	Rees (1979)
Cladonema radiatum	4–5	4–5	1, above filiform tentacles	Schuchert (2006)
Cladonema myersi	4	none	1, below capitate tentacles	Rees (1950)
Cladonema pacificum	4–5	none	1, below capitate tentacles	Hirai (1958); Rees (1982); Takeda et al. (2018)
Cladonema novaezelandiae	not described	not described	not described	Ralph (1953)
Cladonema timmsii	not described	not described	not described	Gershwin and Zeidler (2008)
Cladonema multiramosum sp. nov.	4–6, rarely 10	4–6, rarely 10	1–5, between capitate and filiform tentacles	this study

Table 6. Comparison of the morphology of medusae in the genus *Cladonema*

Species	Umbrella			Radial canals		Tentacles			Tentacle bases		Manubrium	Gonads	Oral tentacles	Reference
	Height/mm	Diameter/mm	Apical projection	No.	Branch pattern	No.	Adhesive branches	Stinging branches	Ocelli	Branches				
Cladonema californicm	short than diameter	2–3	–	9, rarely 11	unbranched	9–11	1	2	1; elongated; red	–	beyond bell margin	6, rarely 7, elongated rounded protrusions	6	Rees (1979)
Cladonema radiatum	2–4	2–3	+/–	7–11	straight or bifurcating near origin	8–10	1–4	3–10	1, red-brown, black, dark red	–	not extending beyond bell margin	4–6 gastric pouches, encircling upper 2/3 of the manubrium	4–7	Raghu (1961); Schuchert (1996, 2006)
Cladonema myersi	not described	not described	–	7, rarely 5–6	unbranched	7	3	7	1; reddish	–	half of bell cavity	not described	6	Rees (1950); Rees (1982)
Cladonema pacificum	2–3	2.2	–	9	straight or bifurcating near origin	9	4	3–6	1; deep purple, nearly black	–	extending beyond bell margin	around the whole manubrium	6	Takeda et al. (2018)
Cladonema novaezelandiae	not described	1.5	not described	7–8	most straight and 1 branched	9	⩽7	⩽10	1	–	not described	6 pouches,	6	Ralph (1953); Schuchert (1996)
Cladonema timmsii	2	2	–	9	unbranched	9	5–7	6–8	1; dark red	–	not extending beyond bell margin	female: encircling upper half of the manubrium, greatly swollen, without pouches; male: 6 radially arranged pouches	6	Gershwin and Zeidler (2008)
Cladonema multiramosum sp. nov.	1.36–1.95	1.57–2.26	+	8–11	straight or bifurcating near origin; some with serval tiny branches on top	8–11	8–24, rarely 5–7	3–6	1, rarely 2; black	+/–	extending beyond bell margin or not	around upper 1/2–1 (mean 4/5) of the manubrium	5–8	this study

Note: + means present; –, absent.

Table 7. Nematocysts (μm) of *Cladonema* species

Stage	Type	*Cladonema multiramosum* sp. nov.	*Cladonema radiatum*	*Cladonema californicm*	*Cladonema pacificum*
Polyp	Stenoteles	(10.2–18.6)×(6.5–10.9)	(11–17)×(8–10)	(14–18.5)×(10–12) 28×18	(16.5–20)×(11.5–12) (13–14)×8
	Mastigophores	(11.1–14.3)×(3.3–4.7)	(10–12)×(3.5–4)	absent	absent
Medusa	Stenoteles	(7.5–18.5)×(4.6–11.9)	(13–16)×(9–10) (9.5–11)×(5–8.5)	(21–23)×(14–16) (14–18)×(9.5–11)	(20.5–23)×(13.5–14) (11.5–14)×(7–8)
	Desmonemes	(6.2–9.0)×(3.3–5.1)	(9–12)×(3.5–5)	(9–11)×(4.5–5)	(9–10)×4
Reference		this study	Schuchert (1996)	Rees (1979)	Rees (1982)

tiramosum sp. nov. from other *Cladonema* medusae by the number of stinging branches alone due to their intersection (Table 6). The manubrium of *C. multiramosum* sp. nov. is flexible in length and could extend beyond the bell margin. In addition, there are 5–8 pouches in the manubrium of *C. multiramosum* sp. nov., similar to the number of pouches in other *Cladonema* medusae except for *C. pacificum* and *C. timmsii* females, which lack pouches in the manubrium (Rees, 1982; Gershwin and Zeidler, 2008; Takeda et al., 2018). *Cladonema multiramosum* sp. nov. presents 5–8 bulbous nematocyst clusters as oral tentacles, similar to other *Cladonema* medusae. The gonads encircle the manubrium, including the pouches occupying the upper 1/2–1 (mean of 4/5) of the manubrium in *C. multiramosum* sp. nov., which occupies the upper 2/3 of *C. radiatum* (Schuchert, 2006), the upper 1/2 of *C. timmsii* females (Gershwin and Zeidler, 2008), and the whole manubrium in *C. pacificum* (Bouillon et al., 1988).

Furthermore, the newly released medusa of *C. multiramosum* sp. nov. resembles *C. pacificum* (Hirai, 1958), *C. californicum* (Rees, 1979), and *C. radiatum* (Schuchert, 1996), and differs from *C. myersi* in that it owns one adhesive branch, while *C. myersi* has two (Rees, 1950). In *C. multiramosum* sp. nov., both the numbers of stinging and adhesive branches increases as the medusae develop. Thus, it is difficult to identify young medusae in the genus *Cladonema*.

As for the nematocysts, *C. multiramosum* sp. nov. possesses stenoteles and desmonemes of variable sizes, as well as in *C. radiatum*, *C. californicum*, and *C. pacificum* (Table 7). A small number of mastigophores exist in the polyps of both *C. multiramosum* sp. nov. and *C. radiatum*, which are unknown in *C. californicum* and *C. pacificum*. Generally, the morphological characteristics of the nematocysts are taxonomically useful for many groups of hydromedusae (Östman, 1979, 1982, 1987); the nematocyst types may assist in classifying *Cladonema* polyps. However, the sizes of the nematocysts are variable, generally larger in mature medusae than in young medusae of *C. californicum* and *C. pacificum* (Rees, 1979, 1982). In addition, some morphological characteristics of the nematocysts are plastic in some hydromedusae (Östman, 1987). Thus, the morphological characteristics of the nematocysts are not suitable to distinguish *Cladonema* species.

Due to lacking information on the hydroid stages and nematocysts in some *Cladonema* species, the taxonomy of the genus *Cladonema* should be predominantly based on the morphological characteristics of the medusa stage, as discussed above.

Key to the accepted nominal species of the genus *Cladonema*

1a With 7, rarely 5–6 marginal tentacles.....*C. myersi*
1b With 8–11 marginal tentacles.....2
2a Stinging branches all emitting directly from tentacle bulb.....3
2b Stinging branches growing from the main branch in turn as side-branches..... 5
3a Most radial canals straight and one-branched...*C. novaezelandiae*
3b Radial canals straight.....4
4a Tentacles with one adhesive branches and two stinging branches.....*C. californicum*
4b Tentacles with 5–7 adhesive branches and 6–8 stinging branches..... *C. timmsii*
5a Manubrium without pouches.....*C. pacificum*
5b Manubrium with pouches.....6
6a Tentacles with 1–4 adhesive branches and 3–10 stinging branches.....*C. radiatum*
6b Tentacles with 8–24 adhesive branches (rarely 5–7) and 3–6 stinging branches.....*C. multiramosum* sp. nov.

4.2 *Molecular phylogenetic analysis*

The results of both genetic divergences and topologies support the hypothesis that *C. multiramosum* sp. nov. is a distinct species. In Fig. 4, *C. multiramosum* sp. nov. forms a sister lineage to *Cladonema* sp. (MT709261), and their genetic divergences (0.091–0.093) are within the intra-genus distances (0.062–0.236) reported in China (Zheng et al., 2014). *Cladonema radiatum* emerges as a sister to *C. multiramosum* sp. nov. (Fig. 4), and they share the most morphological similarities in both polyps and medusa in the genus *Cladonema*, as discussed above.

Our phylogeny of Cladonematidae (Fig. 4) enriches the Cladonematidae clade in the phylogenetics of Capitata based on 16S (Nawrocki et al., 2010). Except *Cladonema* sp. (AM088484), *Cladonema* medusae were recovered as monophyletic with strong support and as a sister to *S. wellingtoni* (Fig. 4), which could be supported by classic relationships relying on morphology (Schuchert, 2006). Although *Cladonema* sp. (AM088484) shows a similar appearance to *C. radiatum* medusa, it presents large genetic distances (more than 0.25) with *C. radiatum* from the Atlantic and Mediterranean (Schuchert, 2006). In current study, *Cladonema* sp. (AM088484) also showed large genetic divergences with other medusae in Cladonematidae (0.239–0.327), with outgroups (Corynidae medusae: 0.282–0.302), which are almost larger than the intra-family distance (0.073–0.287) reported in China (Zheng et al., 2014). Thus, *Cladonema* sp. (AM088484) only formed a sister lineage to *Eleutheria claparedii* with weak support (Fig. 4). In addition, the 16S sequences of only nine species have been reported for Cladonematidae, occupying approximately half the valid species in this family (Schuchert, 2021). The phylogenetic tree will be revised with further information in the future.

Acknowledgements

We thank Ruiting Li for her assistance in the culture of *C. multiramosum*. We thank editors and reviewers for their comments and suggestions on this manuscript. We would like to thank Editage (www.editage.cn) for English language editing.

References

Bouillon J, Boero F, Seghers G. 1988. Notes additionelles sur les Hydroméduses de la mer de Bismarck (Hydrozoa-Cnidaria) II. Indo-Malayan Zoology, 5: 87–99

Bouillon J, Gravili C, Pagès F, et al. 2006. An introduction to Hydrozoa. Memoires du Museum National d'Histoire Naturelle, 194: 591

Bucklin A, Hopcroft R R, Kosobokova K N, et al. 2010a. DNA barcoding of Arctic Ocean holozooplankton for species identification and recognition. Deep-Sea Research Part II: Topical Studies in Oceanography, 57(1–2): 40–48

Bucklin A, Ortman B D, Jennings R M, et al. 2010b. A "Rosetta Stone" for metazoan zooplankton: DNA barcode analysis of species diversity of the Sargasso Sea (Northwest Atlantic Ocean). Deep-Sea Research Part II: Topical Studies in Oceanography, 57(24–26): 2234–2247

Cedeño-Posso C. 2014. First record of the genus *Cladonema* (medusae and polyps) in Colombia. Zootaxa, 3793(5): 597–599, doi: 10.11646/zootaxa.3793.5.8

Ender A, Schierwater B. 2003. Placozoa are not derived cnidarians: evidence from molecular morphology. Molecular Biology and Evolution, 20(1): 130–134

Farias G B, Leitão S N, De Castro Melo P A M, et al. 2020. First *in situ* record of the medusa stage of *Cladonema radiatum* (Cnidaria: Anthoathecata) in the South Atlantic Ocean. Ocean and Coastal Research, 68: e20349, doi: 10.1590/s2675-28242020068349

Folmer O, Black M B, Hoeh W R, et al. 1994. DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology, 3(5): 294–299

Fujiki A, Hou Shiting, Nakamoto A, et al. 2019. Branching pattern and morphogenesis of medusa tentacles in the jellyfish *Cladonema pacificum* (Hydrozoa, Cnidaria). Zoological Letters, 5(1): 12, doi: 10.1186/s40851-019-0124-4

Fujita S, Kuranaga E, Nakajima Y I. 2019. Cell proliferation controls body size growth, tentacle morphogenesis, and regeneration in hydrozoan jellyfish *Cladonema pacificum*. PeerJ, 7: e7579, doi: 10.7717/peerj.7579

Gershwin L A, Zeidler W. 2008. *Cladonema timmsii*, a new species of hydromedusa (Cnidaria: Hydrozoa) from a salt lake in South Australia. Zootaxa, 1826(1): 59–68, doi: 10.11646/zootaxa.1826.1.4

Ghory F S, Ali Q M, Ahmed Q. 2020. First record of *Cladonema radiatum* Dujardin, 1843 (Hydrozoa: Cladonematidae) from northern Arabian Sea, Pakistan. International Journal of Fisheries and Aquatic Research, 5(3): 14–16

Graziussi D F, Suga H, Schmid V, et al. 2012. The "*Eyes absent*" (*eya*) gene in the eye-bearing Hydrozoan Jellyfish *Cladonema radiatum*: conservation of the retinal determination network. Journal of Experimental Zoology Part B: Molecular and Developmental Evolution, 318(4): 257–267, doi: 10.1002/jez.b.22442

Guindon S, Dufayard J F, Lefort V, et al. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Systematic Biology, 59(3): 307–321, doi: 10.1093/sysbio/syq010

He Jinru, Zheng Lianming, Zhang Wenjing, et al. 2015. Morphology and molecular analyses of a new *Clytia* species (Cnidaria: Hydrozoa: Campanulariidae) from the East China Sea. Journal of the Marine Biological Association of the United Kingdom, 95(2): 289–300, doi: 10.1017/S0025315414000836

Hirai E. 1958. On the species of *Cladonema radiatum* var. *mayeri* Perkins. Bulletin of the Marine Biological Station of Asamushi, 9(1): 23–25

Lindner A, Govindarajan A F, Migotto A E. 2011. Cryptic species, life cycles, and the phylogeny of *Clytia* (Cnidaria: Hydrozoa: Campanulariidae). Zootaxa, 2980(1): 23–36, doi: 10.11646/zootaxa.2980.1.2

Nawrocki A M, Schuchert P, Cartwright P. 2010. Phylogenetics and evolution of Capitata (Cnidaria: Hydrozoa), and the systematics of Corynidae. Zoologica Scripta, 39(3): 290–304, doi: 10.1111/j.1463-6409.2009.00419.x

Östman C. 1979. Two types of nematocysts in Campanulariidae (Cnidaria, Hydrozoa) studied by light and scanning electron microscopy. Zoologica Scripta, 8(1–4): 5–12

Östman C. 1982. Nematocysts and taxonomy in *Laomedea, Gonothyraea* and *Obelia* (Hydrozoa, Campanulariidae). Zoologica Scripta, 11(4): 227–241, doi: 10.1111/j.1463-6409.1982.tb00536.x

Östman C. 1987. New techniques and old problems in hydrozoan systematics. In: Bouillon J, Boero F, Cicogna F, et al, eds. Modern Trends in the Systematics, Ecology, and Evolution of Hydroids and Hydromedusae. Oxford: Clarendon Press, 67–82

Östman C. 2000. A guideline to nematocyst nomenclature and classification, and some notes on the systematic value of nematocysts. Scientia Marina, 64(S1): 31–46, doi: 10.3989/scimar.2000.64s131

Purcell J E. 2018. Successes and challenges in jellyfish ecology: examples from *Aequorea* spp. Marine Ecology Progress Series, 591: 7–27, doi: 10.3354/meps12213

Ralph P M. 1953. A guide to the athecate (gymnoblastic) hydroids and medusae of New Zealand. Tuatara, 5(2): 59–75

Raghu P R, Tampi P, Durve V. 1961. Note on the occurrence of the Anthomedusa cladonema in the Indian region. Journal of the Marine Biological Association of India, 3(1–2): 251–252

Rees W J. 1950. On *Cladonema myersi*, a new species of hydroid from the Californian Coast. Proceedings of the Zoological Society of London, 119(4): 861–865

Rees J T. 1979. The polyp and immature medusa stages of *Cladonema californicum*, Hyman, 1947 (Anthomedusae: Cladonemidae) with biological notes and a discussion of the taxonomy of the genus *Cladonema*. Journal of Natural History, 13(3): 295–302, doi: 10.1080/00222937900770231

Rees J T. 1982. The hydrozoan *Cladonema* in California: a possible introduction from East Asia. Pacific Science, 36(4): 439–444

Schuchert P. 1996. The marine fauna of New Zealand: athecate hydroids and their medusae (Cnidaria: Hydrozoa). New Zealand Oceanographic Institute Memoir, 106: 1–159

Schuchert P. 2006. The European athecate hydroids and their medusae (Hydrozoa, Cnidaria): Capitata part 1. Revue suisse de Zoologie, 113(2): 325–410.

Schuchert P. 2016. The polyps of *Oceania armata* identified by DNA barcoding (Cnidaria, Hydrozoa). Zootaxa, 4175(6): 539–555, doi: 10.11646/zootaxa.4175.6.3

Schuchert P. 2021. World Hydrozoa Database. *Cladonema* Dujardin, 1843. http://marinespecies.org/aphia.php?p=taxdetails&id=117049[2021-09-14]

Schuchert P, Hosia A, Leclère L. 2017. Identification of the polyp stage of three leptomedusa species using DNA barcoding. Revue Suisse de Zoologie, 124(1): 167–182

Takeda N, Deguchi R, Itabashi T. 2018. Reproductive strategies in marine Hydrozoan Jellyfish: sexual medusae and asexual polyps. In: Kobayashi K, Kitano T, Iwao Y, et al, eds. Reproductive and Developmental Strategies. Tokyo: Springer, 157–174

Tamura K, Peterson D, Peterson N, et al. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution, 28(10): 2731–2739, doi: 10.1093/molbev/msr121

Xu Zhenzu. 1993. Revisions of nominal species on the Hydromedusae of China sea areas. Journal of Oceanography in Taiwan Strait, 12(3): 197–204

Zheng Lianming, He Jinru, Lin Yuanshao, et al. 2014. 16S rRNA is a better choice than COI for DNA barcoding hydrozoans in the coastal waters of China. Acta Oceanologica Sinica, 33(4): 55–76, doi: 10.1007/s13131-014-0415-8

Zhou Taixuan, Huang Mingxian. 1958. A study on hydromedusae of Chefoo. Acta Zoologica Sinica, 10(2): 173–197

Zhou Konglin, Zheng Lianming, He Jinru, et al. 2013. Detection of a new *Clytia* species (Cnidaria: Hydrozoa: Campanulariidae) with DNA barcoding and life cycle analyses. Journal of the Marine Biological Association of the United Kingdom, 93(8): 2075–2088, doi: 10.1017/S0025315413000969