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Morphology and molecular analyses of a new *Clytia* species (Cnidaria: Hydrozoa: Campanulariidae) from the East China Sea

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The near-cosmopolitan genus Clytia *is abundantly found in coastal waters, but difficulties of identification in this genus make nearly all species records of medusae suspect. Complex life histories, ambiguous taxonomic characters, and phenotypic plasticity pose serious problems for accurate species-level identifications and future revisions of* Clytia *species. In the present study, morphological investigations and molecular analyses of* Clytia *specimens from the coastal waters of the East China Sea revealed* Clytia gulangensis *sp. nov. as a new species. DNA barcoding based on the mitochondrial cytochrome oxidase I (COI) gene supported the new species as a separate species within* Clytia*, and phylogenetic analyses based on mitochondrial 16S rDNA and nuclear 18S rDNA further confirmed this new species to be a distinct lineage. Moreover, detailed observation of medusae and polyps of this species showed sufficient morphological differences from other* Clytia *species for a diagnosis. Our results indicated that life cycle and DNA-based studies should be a standard approach in future biodiversity investigations of* Clytia *species.*

Keywords: *Clytia*, new species, morphology, molecular analysis

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INTRODUCTION

Clytia Lamouroux, 1812 is a typical Campanulariidae (Cnidaria: Hydrozoa: Leptothecata) genus with many species and a near-cosmopolitan distribution. The life cycle of *Clytia* comprises a free swimming medusae stage and a sessile hydroid stage. Medusae of *Clytia* are frequently found in surface coastal waters, while hydroids of this genus are common in shallow-water benthic communities (Cornelius, 1995; Madin *et al.*, 1996; Boero *et al.*, 2005; Lindner *et al.*, 2011; Zhou *et al.*, 2013). With growing attention focused on the ecological impact of jellyfish blooms, these tiny organisms are emerging as an important competitor and predators in the coastal marine ecosystem (Lucas *et al.*, 1995; Bouillon *et al.*, 2006; Gravili *et al.*, 2008; Miglietta *et al.*, 2008). Certain species, e.g. *C. hemisphaerica* (Linnaeus, 1767), have been firmly studied as a basal metazoan model organism to explore developmental mechanisms and resolve basic evolutionary questions (Houliston *et al.*, 2010).

However, species diagnosis in *Clytia* is challenging. Firstly, most species are based either on the hydroid (42 species) or the medusae form (30 species), with only 11 species having had their complete life cycle investigated[1] (Mayer, 1910; Roosen-Runge, 1970; West & Renshaw, 1970; Kubota, 1978a, b; Cornelius, 1995; Pagliara *et al.*, 2000; Lindner & Migotto, 2002; Gravili *et al.*, 2008; Lindner *et al.*, 2011; Zhou *et al.*, 2013). Secondly, diagnostic characters of some species in the genus were based on a single specimen, or sometimes even on an immature animal, e.g. *C. ambigua* (Agassiz & Mayer, 1899) and *C. hexacanalis* (Xu *et al.*, 1991). Thirdly, characters long thought to have taxonomic values tend to be variable between individuals of the same population, at least in certain species (Kubota, 1978a; Bouillon & Boero, 2000). Finally, with the emerging of molecular techniques, cryptic species or even new species are being revealed (Lindner *et al.*, 2011; Zhou *et al.*, 2013).

Molecular-aided species diagnosis and phylogenetic analyses have made remarkable progress in species revisions and reconstruction of the evolution in the related taxa. DNA barcoding based on mitochondrial COI proved to be a useful molecular tool to identify species efficiently and reliably among many animal taxa (Hebert *et al.*, 2003a, b; Bucklin *et al.*, 2010a, b, 2011; Ortman *et al.*, 2010). In the genus *Clytia*, COI was also applied to identify ambiguous specimens (Laakmann & Holst, 2014) and detect cryptic or new species

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[1]Data stated here are based on the valid *Clytia* species list from the World Register of Marine Species (reference listed below) and publications on life cycle reports herein.

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(Lindner *et al.*, 2011; Zhou *et al.*, 2013). Recently, mitochondrial 16S was proposed as a candidate for hydrozoa barcoding (Moura *et al.*, 2008; Zheng *et al.*, 2014). Meanwhile mitochondrial 16S and COI, nuclear 18S, 28S and internal transcribed spacer 1 (ITS1) genes were used to resolve phylogenetic relationships at various taxonomic levels (Bridge *et al.*, 1995; Schierwater & Ender, 2000; Collins *et al.*, 2005, 2006; Govindarajan *et al.*, 2006; Schuchert, 2014). In this study, morphological observations and molecular analyses were combined to validate the taxonomic position of a new *Clytia* species, *Clytia gulangensis* sp. nov., and thus to promote the revision of the genus *Clytia*.

MATERIALS AND METHODS

Medusae were collected from Xiamen Bay (24.4514°N 118.0753°E), East China Sea, using a plankton net with mesh size of 505 μm on 5 June 2012 (Figure 1). In the laboratory, 22 mature medusae with indistinguishable morphologies were kept in a 20 × 20 × 20 cm glass tank with filtered seawater (filter mesh size: 50 μm). Hydroids developed at the bottom of the aquaria after five days and they were then transferred onto a glass slide in a new tank. Colonies developed from two separate glass slides covered the whole tank surfaces eventually, and were used for life-cycle observation, respectively. Both medusae and hydroids were fed with *Artemia* sp. nauplii daily with water changed every other day after feeding. Water temperature was kept at 22 ± 3°C and salinity at 31 ± 2.

Morphological measurements were accomplished with Zeiss SteREO Discovery V12 and Olympus BX51 microscope. Individuals intended for image documenting were acclimated with 3% $MgCl_2$, specimens for morphological preservation were fixed in 5% formalin and those for DNA preservation were fixed in 95% ethanol. Fresh tissue was stimulated in 1% SDS (sodium dodecyl sulphate) for nematocysts type and distribution detection, and nematocysts nomenclature followed that of Östman (1999, 2000).

Total DNA was extracted from both medusa and hydroid with a modified phenol-chloroform extraction method (Zheng *et al.*, 2009); mitochondrial COI (primer: HCO2198-taaacttcagggtgaccaaaaaatca, LCO1490-gtcaacaaatcataaagatattgg; Folmer *et al.*, 1994) and 16S (primer: 16SH-cataattcaacatcgagg, 16SL-gactgtttaccaaaaacata; Ender & Schierwater, 2003), nuclear 18S (primer: 18SF-gctgtatgtactgtgaaactgcg, 18SR-cacctacggaaaccttgttacgac; Leclère *et al.*, 2009) and 28S (primer: 28S1F-tcccctagtaacggcgagtgaagcg, 28S1R-gagccaatccttwtcccgargtt, 28S2F-gacagcaggacggtggycatgg, 28S2R-ttcygacttagaggcgttcag; Medina *et al.*, 2001; Leclère *et al.*, 2009) gene fragments were amplified according to the references herein. Purified PCR products were sequenced by Sangon Biotech on 3730xl DNA Analyzer with BigDye terminator v.3.1.

Sequences were checked manually based on chromatogram files, aligned using the NCBI Nucleotide Blast (BLASTn) program to confirm the validity, and submitted to the NCBI GenBank database. GenBank Accession numbers of *C. gulangensis* sp. nov. are KF962086–KF962090 (hydroid), KF962091–KF962095 (medusae from field), and KF962096–KF962100 (medusae from culture) for COI; KF962425–KF962429 (hydroid), KF962430–KF962434 (medusae from field), and KF962435–KF962439 (medusae from culture) for 16S; KF962218–KF962222 (hydroid), KF962223–KF962227 (medusae from field), and KF962228–KF962232 (medusae from culture) for 18S; and KF962318–KF962322 (hydroid) and KF962323–KF962327 (medusae from field), and KF962328–KF962332 (medusae from culture) for 28S, respectively.

Multiple sequences were aligned using ClustalX V2.1 (Larkin *et al.*, 2007), genetic distance was determined by MEGA 5.2 (Tamura *et al.*, 2011) with Kimura-2-Parameter model, and phylogenetic analyses were performed using PAUP* 4.0b10 (Swofford, 2002) and MEGA 5.2 with GTR + G + I model which was suggested as optimal substitution model by the built-in model test module. For DNA barcoding purpose, Kimura 2-parameter genetic distance and neighbour-joining tree were generated from all *Clytia* COI sequences available in GenBank, with Leptothecata *Aequorea conica* Browne, 1905 and *Gangliostoma guangdongensis* Xu, 1983 selected as outgroups. For phylogenetic analyses of *Clytia*, maximum likelihood and maximum parsimony analyses were conducted for 16S and 18S individually and for both genes combined; sequences of both genes from all *Clytia* species and other representative campanulariids in GenBank were included; and *Calycella syringa* Linnaeus, 1767 and *Opercularella pumila* Clark, 1875 (accepted as *Campanulina pumila* Clark, 1875) were selected as outgroups (Govindarajan *et al.*, 2006; Lindner *et al.*, 2011). We preferred the results obtained with likelihood analyses because of known problems of parsimony with rate variation (Govindarajan *et al.*, 2006). Though nuclear 28S rDNA was proved to be an informative marker for both species revision and evolution analysis purpose (e.g. Evans *et al.*, 2008; Leclère *et al.*, 2009), it was not used in this study as not enough data from *Clytia* species were available. Taxa employed in this study and GenBank accession numbers are listed in Table S1.

RESULTS

SYSTEMATICS

Phylum CNIDARIA
Class HYDROZOA Owen, 1843
Order LEPTOTHECATA Cornelius, 1992
Family CAMPANULARIIDAE Johnston, 1836
Genus *Clytia* Lamouroux, 1812
Clytia gulangensis sp. nov. Jinru He & Lianming Zheng
(Figures 2–4; Tables 1–3)

MATERIAL EXAMINED

Holotype: XMBCG01, male medusa, diameter 6.43 mm, height 2.36 mm.

Paratypes: XMBCG02, male medusa, diameter 6.82 mm, height 2.62 mm; XMBCG03, male medusa, diameter 10.70 mm, height 3.62 mm; XMBCG20, polyp with both trophosome and mature gonosome.

Nontype material: XMBCG07, female medusa, diameter 5.78 mm, height 2.54 mm; XMBCG14, female medusa, diameter 6.00 mm, height 2.52 mm; XMBCG05, polyp with both trophosome and immature gonosome.

The medusae specimens XMBCG01 and XMBCG02 were collected in Xiamen Bay, China, 24°27′5″N 118°4′31″E, 5 June 2012. XMBCG03, XMBCG07, XMBCG14 (medusa)

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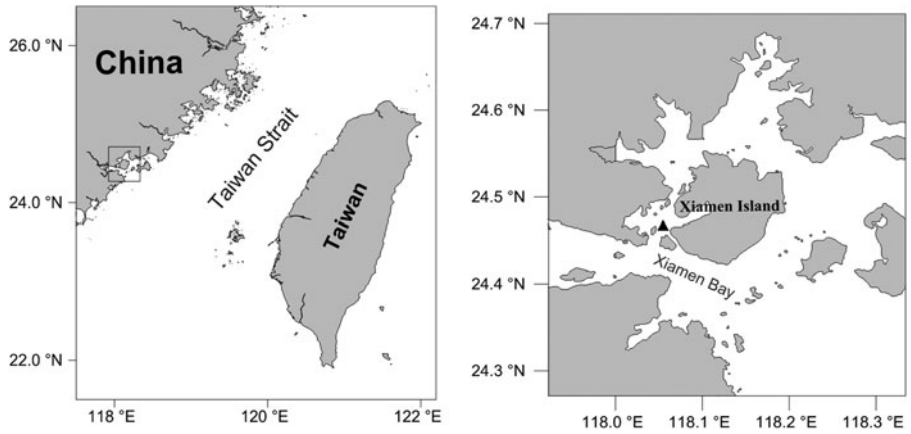

Fig. 1. Sampling site of *Clytia gulangensis* sp. nov.

and XMBCG05, XMBCG20 (polyps) were obtained from individuals cultured in the laboratory. All type specimens are deposited in the Department of Marine Biological Science and Technology, College of Ocean and Earth Sciences, Xiamen University, China.

ETYMOLOGY

Clytia gulangensis sp. nov. is named after Gulang Island around which the specimens were collected.

DIAGNOSIS

The stolonal polyps, the campanulate hydrothecae, and free medusae with a normal velum, without cirri or excretory papillae identify the animals as members of the genus *Clytia*.

Clytia gulangensis sp. nov. is distinguished from its congeners and other campanulariids by the combination of the following characters:

Polyp: stems monosiphonic, sometimes polysiphonic, branching irregularly 2–3 times. Hydrotheca elongate campanulate, about three times as long as wide, with 8–12 blunt, triangular cusps, slightly asymmetrical, separated by deep, rounded embayments, without inward folds. Gonotheca on hydrorhiza and pedicel, club-shaped, somewhat pod-like, with neck, stalk short with indistinct 1–3 annulations, with smooth walls, forming one to two rows of up to six medusae buds. Mature ones with neck just below the aperture. B-type microbasic mastigophores 7.98–8.48 μm long and 2.16–2.21 μm wide.

Adult medusa: umbrella flatter than a hemisphere, 6.2–10.5 mm in diameter, up to 36 tentacles, with 1–2 statocysts between successive tentacles, each containing a single statolith, rarely two. Gonads linear, more wavy band-like when mature, covering 3/5–4/5 of the radial canal, leaving spaces at both ends. Ic-type isorhizas 7.21–7.50 μm long and 2.54–2.58 μm wide.

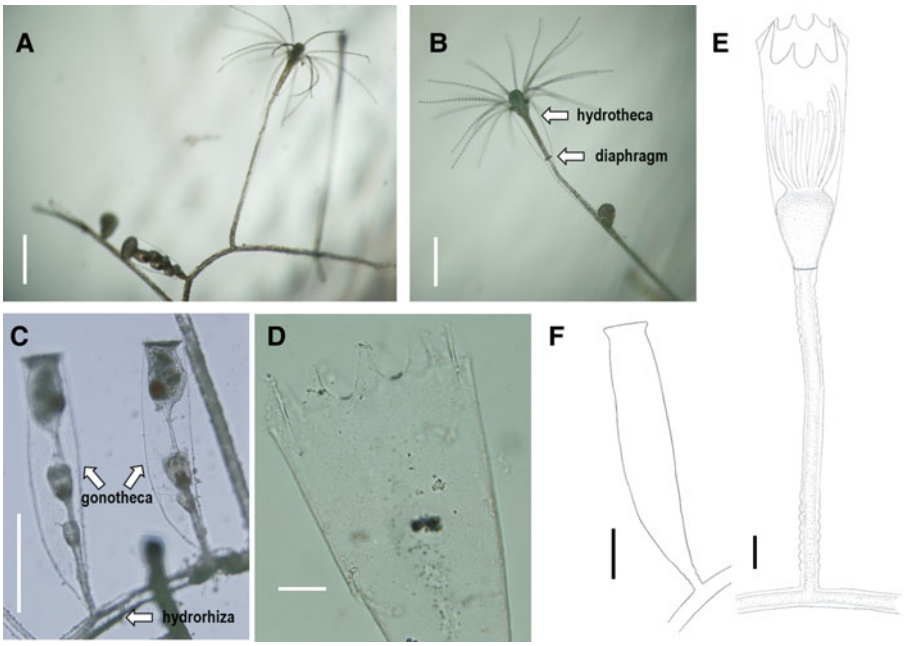

Fig. 2. Hydroids of *Clytia gulangensis* sp. nov.: (A) colony; (B) hydranth; (C) gonotheca with medusae buds; (D) hydrothecal margin with cusps; (E) illustration of a trophotheca; (F) illustration of a gonotheca. Scale bars: A–C, 0.5 mm; D, 0.1 mm; E, 0.5 mm; F, 0.25 mm.

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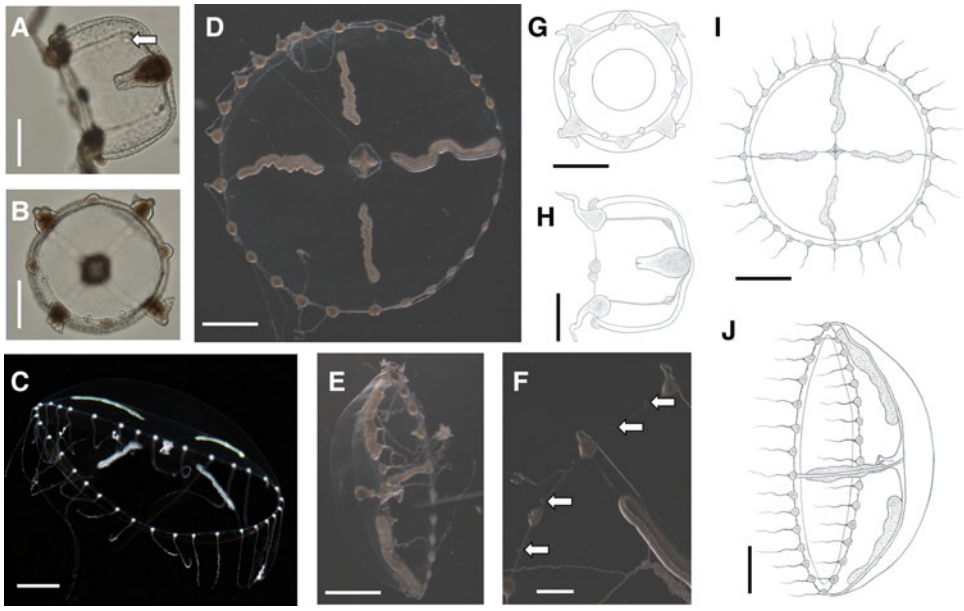

Fig. 3. Medusae of *Clytia gulangensis* sp. nov.: (A) newly released medusa, side view, white arrow: gonad; (B) newly released medusa, oral view; (C) mature medusae (60 days), side view; (D) mature medusae (35 days), oral view; (E) mature medusae (35 days), side view; (F) margin of mature medusa, white arrow: statocysts with statoliths; (G, H) illustration of newly released medusa; (I, J) illustration of mature medusa. Scale bars: A, B, 0.2 mm; C–E, 1.5 mm; F, 0.5 mm; G, H, 0.2 mm; I, 0.3 mm; J, 0.2 mm.

DESCRIPTION

Hydroid

Colonies stolonal or with erect stems branching 2–3 times irregularly. Branches given off upwardly from stem; pedicel up to 5.9 mm high, smooth, with 9–17 proximal and 5–10 distal annuli. Creeping hydrorhiza slightly annulated occasionally at the junction where branches occurred (Figure 2A, E).

Hydrothecae elongate campanulate, with thin perisarc and smooth walls, about 3 times as long as wide (0.53–1.02 mm long and 0.18–0.33 mm wide at aperture); rim with 8–12 blunt, slightly asymmetrical, triangular or pyramidal cusps, separated by deep, rounded embayments, without inward folds. Hydrothecal diaphragm thin, near base of hydrotheca; basal chamber 46–89 μm long and 78–114 μm wide at diaphragm. Pedunculated hypostome spherical or oval in oral view. Hydranth with 14–24 filiform tentacles, 0.5–0.8 mm in length (Figure 2B, D; Table 1). Coenosarc whitish.

Gonotheca on stolons and pedicels, or directly on branches, nearly cylindrical in shape. And sometimes pod-like. Gonothecae smooth, about three times as long as wide (0.79–0.90 mm long and 0.26–0.29 mm wide at distal

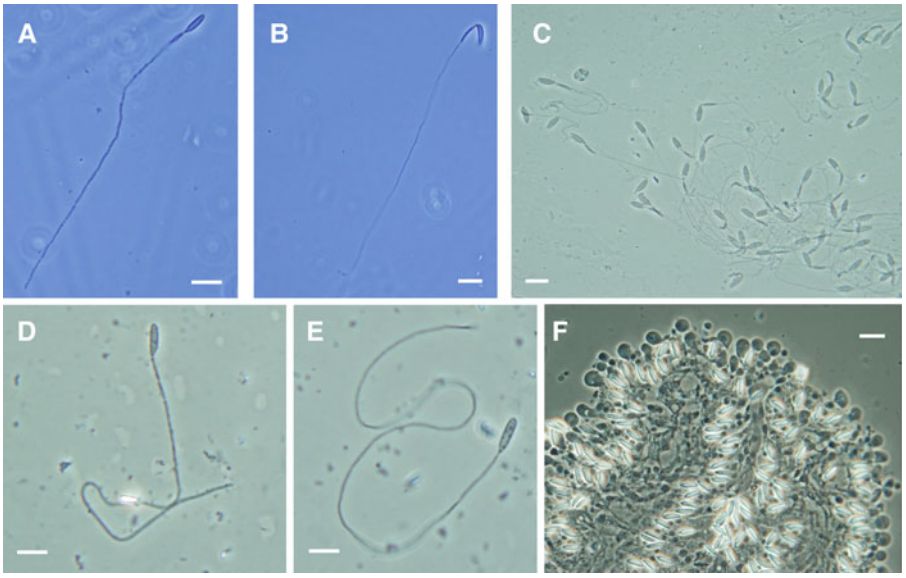

Fig. 4. Nematocysts of *Clytia gulangensis* sp. nov.: (A) A-type microbasic mastigophores from hydranth; (B) B-type microbasic mastigophores from hydranth; (C) A-type microbasic mastigophores from newly released medusae; (D) Ic-type isorhiza from newly released medusae; (E) Ic-type isorhiza from mature medusae; (F) A-type microbasic mastigophores from mature medusae. Scale bars: A–F, 10 μm.

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Table 1. Measurements (mean ± standard deviation (range)) of colonies of *Clytia gulangensis* sp. nov. (N = 30).

Hydrothecal pedicel	
Length (mm)	3.17 ± 1.60 (1.4–5.9)
Width (mm)	0.08 ± 0.01 (0.06–0.10)
Basal annuli	11.45 ± 2.24 (9–17)
Distal annuli	6.68 ± 1.17 (5–10)
Hydrothecae	
Shape	Campanulate
Length (mm)	0.75 ± 0.18 (0.53–1)
Maximum width (mm)	0.24 ± 0.06 (0.18–0.33)
Length: width	3.07 ± 0.12 (2.75–3.28)
Marginal cusp	Asymmetrical, blunted
Number of cusps	8–10
Tentacle	
Number	20.53 ± 3.34 (14–24)
Length (mm)	0.63 ± 0.12 (0.5–0.8)
Gonothecae	
Shape	Cylindrical, with neck
Wall	Thin, smooth
Length (mm)	0.83 ± 0.04 (0.79–0.90)
Width (mm)	0.28 ± 0.01 (0.26–0.29)
Length: width	3.01 ± 0.16 (2.81–3.20)
Basal annuli	0–2, indistinct
Number of medusa	5–7, 2 rows

end); with one side of the wall nearly straight and opposite side contracting down, with constriction below the truncated distal margin, with short stalk, slightly annulated 1–3 times. Up to 6 medusae of 1–2 rows in each gonangium (Figure 2C, F; Table 1).

A- and B-type microbasic mastigophores on hydranth and coenosarcs. Capsules of discharged A-type microbasic mastigophores 6.76 ± 0.60 (6.07–7.82) μm long and 2.00 ± 0.25 (1.75–2.40) μm wide, and B-type microbasic mastigophores 8.23 ± 0.35 (7.98–8.48) μm long and 2.19 ± 0.04 (2.16–2.21) μm wide *in vivo*, respectively. Tubes of discharged A-type microbasic mastigophores form an obtuse angle to the long axis of the capsule, but sometimes coincide with the direction of the latter. Shaft of discharged B-type microbasic mastigophores wider and longer than other types, and the angle between shaft and long axis more obvious (Figure 4A, B; Table 3).

Table 3. Measurements (mean ± standard deviation (range)) of microbasic mastigophore nematocysts of *Clytia gulangensis* sp. nov., in μm. (N = 50).

		Length (μm)	Width (μm)
A-type	Hydroid	6.76 ± 0.60 (6.07–7.82)	2.00 ± 0.25 (1.75–2.40)
	Medusa	7.58 ± 0.40 (6.99–8.15)	2.26 ± 0.17 (2.12–2.54)
B-type	Hydroid	8.23 ± 0.35 (7.98–8.48)	2.19 ± 0.04 (2.16–2.21)
Ic-type	Medusa	7.40 ± 0.20 (7.21–7.50)	2.56 ± 0.03 (2.54–2.58)

Newly-released medusae

Umbrella bell-shaped, somewhat cubic, 0.43–0.58 mm in diameter and 0.39–0.57 mm in height; with ring canal and four radial canals; four prominent perradial bulbs with tentacles and four small interradial developing bulbs; eight adradial statocysts, each containing a single statolith. Gonads on proximal 1/3 of radial canals, oval in shape. Manubrium quadrate, half the height of bell cavity, with slightly recurved lips. Velum broad (Figure 3A, B; Table 2).

Development

Medusae two days after release with eight tentacles and eight statocysts. Umbrella flattened with diameter increasing, gonads extending along radial canals and turned wavy band-like three weeks since release. Tentacle number increased to 30 or more in about 20 days, and statocyst number increased from one to two between two successive tentacles in most cases during growth.

Mature medusae

Medusae grew mature about 35 days after liberation as judged by sperm or egg release. Umbrella flatter than a hemisphere, 6.2–7.0 mm in diameter and 2.2–2.6 mm in height. Jelly thin and flexible. Tentacles 24 to 30 in number, well-developed, with mediate, rounded, basal bulbs. Statocysts 31 to 37 in number, alternate in position with the tentacles,

Table 2. Comparison of morphology (mean ± standard deviation (range)) of *Clytia gulangensis* sp. nov. at successive developing medusae stages, in mm (N = 30 unless otherwise mentioned).

		Newly released stage (0 day)	35 days	60 days (N = 5)
Umbrella	**Shape**	**Bell-shaped**	**Hemispherical**	**Flat**
	Diameter (mm)	0.54 ± 0.06 (0.43–0.58)	6.4 ± 0.50 (6.2–7.0)	10.4 ± 0.36 (9.8–10.9)
	Height (mm)	0.46 ± 0.07 (0.39–0.57)	2.4 ± 0.22 (2.2–2.6)	3.53 ± 0.24 (3.3–3.7)
Gonad	Shape	Oval	Linear	Linear
	Position	Proximal 1/3	Middle 4/5	Middle 3/5
	Length (mm)	0.023 ± 0.00 (0.021–0.023)	2.10 ± 0.60 (1.6–2.8)	3.23 ± 0.35 (2.9–3.6)
	Width (mm)	0.023 ± 0.00 (0.021–0.023)	0.33 ± 0.04 (0.29–0.38)	0.39 ± 0.13 (0.28–0.41)
Mouth and lips		Simple mouth, no apparent lips	Simple, cross	Simple, cross
Manubrium	Height (mm)	0.22 ± 0.01 (0.21–0.23)	0.36 ± 0.09 (0.31–0.44)	0.48 ± 0.15 (0.42–0.53)
	Width (mm)	0.11 ± 0.01 (0.10–0.13)	0.44 ± 0.11 (0.36–0.52)	0.53 ± 0.07 (0.51–0.55)
Tentacle	Number	4	25 ± 2.08 (24–30)	34 ± 1.41 (33–36)
	Bud	4	3 ± 0.58 (2–3)	0
	Basal shape	Round	Triangular, blunted	Triangular, blunted
Statocyst	Number	8	34 ± 3.00 (31–37)	56 ± 1.41 (55–57)
	NBST*	1	1–2, mostly 1	1–2, generally 2
Statolith	Number	1	1, occasionally 2	1, occasionally 2

NBST*, number between successive tentacles.

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rarely two between successive tentacles, each containing a single, spherical concretion. Velum narrow, four straight, slender radial canals and a narrow circular canal. Manubrium short and quadratic in cross-section, four slightly recurved lips. Four gonads situated very close to circular canal, and stretched to gastro-vescular cavity, occupied almost entire radial canals when fully developed, also became more band-like and somewhat contorted. The medusa is transparent with the exception of the manubrium, gonads, and tentacle bulbs, which are somewhat flesh-colour (Figure 3D–F; Table 2).

Further rearing produced medusae with extended umbrella diameter and increased statocyst number. Few medusae with longevity about 60 days measured maximum diameter of 10.9 mm and height of 3.7 mm. Tentacle numbers up to 36 without rudimentary bulbs, and statocysts of 55–57 in total and mostly two between successive tentacles, statolith remained single in each statocyst, rarely two. The wavy banded linear gonads extended about 3/5 of the radial canals (Figure 3C; Table 2).

Variation of nematocysts size among individuals is insignificant and discharged capsule size from young and adult medusae reveal little differences. A-type (7.58 ± 0.40 μm long and 2.26 ± 0.17 μm wide) microbasic mastigophores from medusae are larger than those from hydroid. And Ic-type isorhizas (7.40 ± 0.47 μm long and 2.56 ± 0.03 μm wide) are frequently found in medusae (Figure 4C–F; Table 3).

DISTRIBUTION

This species is only collected in large numbers on the surface in Xiamen Bay, East China Sea. Further field investigations on its distribution and seasonal variation remain unresolved.

BIOLOGICAL NOTES

This new species can be easily reared under conditions described above. And medusae release in large numbers in about every 20 days. When the temperature is maintained at 12–30°C, salinity at 25–35, and fed every 1–3 days, hydroids can survive with at least 1/3 hydranth extending and feeding, while budding could be affected by delayed release. The polyps seem to be rather tolerant to salinity variation (25–40) induced by water evaporation and replenishment of fresh water, while starvation (longer than 5 days) and low temperature (below 10°C) would be a devastating induction to hydranth resting. For medusae, warm temperature (25–30°C), optimal salinity (28–32) and daily feeding would be necessary, and water quality should be secured to avoid unexpected death.

REMARKS

Hydroids of *C. gulangensis* sp. nov. resemble *C. delicatula* (Thornely, 1900), *C. elongata* (Marktanner-Turneretscher, 1890), *C. elsaeoswaldae* (Stechow, 1914), *C. gracilis* (Sars, 1850), *C. gregaria* (Agassiz, 1862), *C. linearis* (Thorneley, 1900) and *C. tottoni* (Leloup, 1935) as all of them have elongate campanulate hydrothecae and smooth gonothecal walls. Both *C. gregaria* and *C. linearis* show signs of inward folds at the hydrothecal embayments (Roosen-Runge, 1970; Lindner & Migotto, 2002; Schuchert, 2003), which is not the case in our species; *C. elsaeoswaldae*, *C. gracilis* and *C. tottoni* are all similar to *C. gulangensis* sp. nov. in having inclined hydrothecal cusps, while *C. elsaeoswaldae* have slightly undulated gonothecal walls, *C. gracilis* have tilted cusps with one side almost vertical and the other oblique, with also slightly everted embayment margin, and *C. tottoni* have cusps projecting inwardly, instead of inclined pyramidal cusps and smooth embayments in our species (Cornelius, 1995; Schuchert, 2003; Galea, 2010; Lindner *et al.*, 2011); the ratio of hydrothecal length to width is about 3 (2.75–3.28) in our species, while hydrothecae of *C. delicatula*, *C. elongata*, and *C. gracilis* are all about 2 times as long as wide, moreover, *C. delicatula* have deeply-cut, acute cusps (Hiro, 1939; Kubota, 1978a), hydranth of *C. elongata* have just about 10 tentacles, instead of 14–24 in our species (Vervoort & Watson, 2003). The B-type microbasic mastigophores of *C. gulangensis* sp. nov. (7.98–8.48 × 2.16–2.21 μm) is much smaller in size compared to other *Clytia* species (10.0–24.0 × 2.5–5.5 μm) reported, with the exception of *C. noliformis* (McCrady, 1859) (6.5–7.0 × 2.0–2.5) (Östman, 1979a, b, 1999) (Table 3).

Medusae of the present species resemble those of *C. attenuata* (Calkins, 1899), *C. brunescens* (Bigelow, 1904), *C. gregaria*, *C. hemisphaerica*, *C. languida* (Agassiz, 1862), *C. lomae* (Torrey, 1909), *C. macrogonia* (Bouillon, 1984), *C. malayense* (Kramp, 1961) and *C. uchidai* (Kramp, 1961) in having about 30 marginal tentacles and 1–2 statocysts between successive tentacles, but differ in shape and situation of gonads. *Clytia attenuata* is considered conspecific with *C. gracilis* (Calder, 1991), or C. *hemisphaerica* (Cornelius, 1982; Bouillon *et al.*, 2006), and a life-cycle study reported the gonads of mature *C. attenuata* to be oval to sacciform (West & Renshaw, 1970), while redescription of *C. gracilis* from north-west European waters revealed that medusae of this species have up to 16 tentacles (Cornelius, 1995). *Clytia brunescens* bears thick and prominent gonads which are nearly hemispherical and occupying proximal third of radial canal (Bigelow, 1904); *C. gregaria*, *C. hemisphaerica*, *C. lomae*, *C. malayense* and *C. uchidai* all have oval to linear ovaries which extend distal half of radial canal (Agassiz, 1862; Torrey, 1909; Kramp, 1961; Kubota, 1978b); *C. languida* bears linear ovaries nearly covering the entire radial canal, but statocysts between every two tentacles are 2–3 in number (Agassiz, 1862); *C. macrogonia* has cylindrical gonads extending almost entire radial canal which is much more prominent than those of our species, and its manubrium is cruciform with rounded perradial lobes which are absent in *C. gulangensis* sp. nov. (Bouillon, 1984; Bouillon *et al.*, 2004; Du *et al.*, 2012) (Table 4).

Though sharing a similar shape of umbrella and the approximate number of tentacles in medusae, *C. gulangensis* sp. nov. is also different from *C. xiamenensis* (Zhou *et al.*, 2013), which was described recently from the same area from both hydroid and medusae stages. In *C. xiamenensis*, gonothecal walls are undulated, statocysts being 0–3 in number, and gonads occupying distal half of radial canal (instead of smooth gonothecal walls, 1–2 nematocysts between successive tentacles, and elongated wavy gonads in our species); the novel LA-type microbasic mastigophores from *C. xiamenensis* are never found in our individuals, and B-type microbasic mastigophores of *C. gulangensis* sp. nov. are much smaller than that of *C. xiamenensis* (Zhou *et al.*, 2013) (Table 4).

DNA BARCODING AND PHYLOGENETICS

The COI alignment for DNA barcoding analysis included 95 operational taxonomic units (OTUs) and 669 base pairs. Sequence divergence (measured as K2P genetic distance) of

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Table 4. General features of some similar species of *Clytia*.

		C. gulangensis	*C. gracilis*	*C. hemisphaerica*	*C. linearis*	*C. xiamenensis*
Colony		Branching	Rarely branch	Branching	Branching	Rarely branch
Hydrothecae	Length: width	~3	~2	~2	2–3	~2
	Cusp	Blunt, inclined	Pointed, inclined	Triangular	Inward folds	Pointed, inclined
Gonothecae	Wall	Smooth	Smooth	Corrugated	Smooth	Undulated
	Pedicel	Short annulated	Short annulated	Annulated	Annulated	Short annulated
Mature medusae	Diameter (mm)	6.2–10.5	6–8	<20	2.5–3.6	5.7–9.1
	Tentacle	28–36	<16	<32	20–29	19–44
	Statocyst	1–2	1–2	1–3	1–2	0–3
	Gonad	Middle 4/5	Oval	Elongate, distal	Oval, distal	Linear, distal
Reference		Present study	Cornelius (1995)	Cornelius (1995)	Lindner & Migotto (2002)	Zhou *et al.* (2013)

COI between individuals of *C. gulangensis* sp. nov. ranged from 0 to 0.003. For the genus *Clytia*, intra-specific genetic distance varied from 0 (multiple species, e.g. *C. folleata* (McCrady, 1859)) to 0.014 (*C. hemisphaerica*); inter-specific genetic distance ranged from 0.049 (between *C. elsaeoswaldae* and *Clytia* sp. 701AC) to 0.257 (between *Clytia* cf. *gracilis* sp. D and *C. hummelincki* (Leloup, 1935)). For *C. gulangensis* sp. nov., minimum genetic distance (0.062) was observed in comparison to *Clytia* cf. *gracilis* sp. B; maximum genetic distance (0.198) was observed in comparison to *Clytia* cf. *gracilis* sp. D (Table S2). Thus, a barcoding gap (Meyer & Paulay, 2005) was confirmed both in the genus *Clytia* and between *C. gulangensis* sp. nov. and all the other *Clytia* species, respectively. In the present study, genetic distance between *C. gulangensis* sp. nov. and the other four species collected in sympatry, ranged from 0.071 to 0.110, also showed obvious barcoding gaps (details in Tables S1 and S2). Neighbour-joining topology of all *Clytia* and other representative Campanulariidae failed to recover a monophyletic *Clytia* as certain sequences of *C. linearis*, *C. hummelincki* and *Clytia*. cf. *gracilis* sp. D were placed outside the Clytiinae clade, but still revealed an independent clade of *C. gulangensis* sp. nov., which supported the validity of this new species (Figure 5).

Maximum likelihood topology based on the 16S (Figure S1), 18S (Figure S2) and 16S plus 18S (Figure 6) dataset all suggested a monophyletic *C. gulangensis* sp. nov. clade, which further confirmed the separation from all known *Clytia* sequences. Taxa incorporated into the Campanulariidae phylogenetic analysis were adjusted to include representatives from subfamily Obeliinae and Campanulariinae, along with all Clytiinae (genus *Clytia*) sequences available on GenBank. Though two sequences (*C. hemisphaerica* FJ550601; *C. noliformis* EU272611) which have only 18S entries and five sequences (*C. elsaeoswaldae* DQ068064; *C. hemisphaerica* EU999221; *C. hemisphaerica* HM053545; *Clytia* sp. AY800195; *C. viridicans* (Leuckart, 1956) AY346365) which have only 16S entries were excluded in the final alignment, 11 *Clytia* species with morphological descriptions were included. Maximum likelihood analyses for 16S failed to recover a monophyletic Clytiinae, and 18S failed to reveal a monophyletic Obeliinae, respectively. The 16S plus 18S alignment for phylogenetic analysis, which included 71 OTUs and 2252 base pairs, revealed both monophyletic Obeliinae and Campanulariinae, and thus better resolved the relationships among Campanulariidae relatively. With *C. hummelincki* turning out to be a sister taxon to Obeliinae plus *Clytia* (with the only exception of *C. hummelincki*) clade, Clytiinae failed to form a monophyletic lineage yet. And the monophyletic *C. gulangensis* sp. nov. clade was deeply rooted in the main *Clytia* clade (Figure 6).

DISCUSSION

Life-cycle investigations are essential for species diagnosis and taxonomic revision in the genus *Clytia*. Life-cycle studies provide valuable information on variations of taxonomic characters by investigating the developmental process (e.g. Kubota, 1978b), and helped uncover cryptic or new species by supplementing novel characters in another stage (Lindner *et al.*, 2011; Zhou *et al.*, 2013). Currently, 61 *Clytia* species have been recognized, of which 60 species are listed in the World Register of Marine Species (WoRMS) (Schuchert, 2013) and *C. xiamenensis* was described recently (Zhou *et al.*, 2013). Among all those species recorded, only 11 species have their life cycle investigated, but for *C. hummelincki* the mature medusa remains unknown (Gravili *et al.*, 2008). With most species being morphologically diagnosed either by their polyp or medusae stage, the gap between diagnoses based on only one of the stages definitely contributed to the inflation of synonyms in this genus (e.g. Calder, 1991; Schuchert, 1998). In the present study, the fixed hydrothecal length to width ratio, blunt and asymmetric cusps, smooth gonothecal walls, in association with linear, extended gonads and smaller B-type microbasic mastigophores supported *C. gulangensis* sp. nov. to be unique compared to all the existing *Clytia* species. However, diagnosis characters for either medusae or hydroid alone are quite ambiguous. With tentacle numbers ranged from 24 to 36 and statocysts between successive tentacles about 1–2, medusae of *C. gulangensis* sp. nov. from field plankton specimens can be easily miss-identified to other related *Clytia*, e.g. *C. hemisphaerica*, *C. gracilis* and *C. linearis*. Meanwhile, pieces of colony bearing a single campanulated hydrotheca with blunt, triangular cusps or a smooth, cylindrical gonotheca cannot be attributed exclusively to an exact species, either. Thus, careful investigations on life cycle description would be essential to uncover detailed diagnosis characters both from hydroid and medusae to detect novel species and help resolve species revision (Zhou *et al.*, 2013).

DNA barcoding offers great help for *Clytia* biodiversity studies by identifying species easily and quickly. While morphological studies offer great details about taxonomic placement for type specimens, immature individuals or fragments cannot be identified reliably using morphology alone (Bouillon & Boero, 2000). For 17 *Clytia* species recorded

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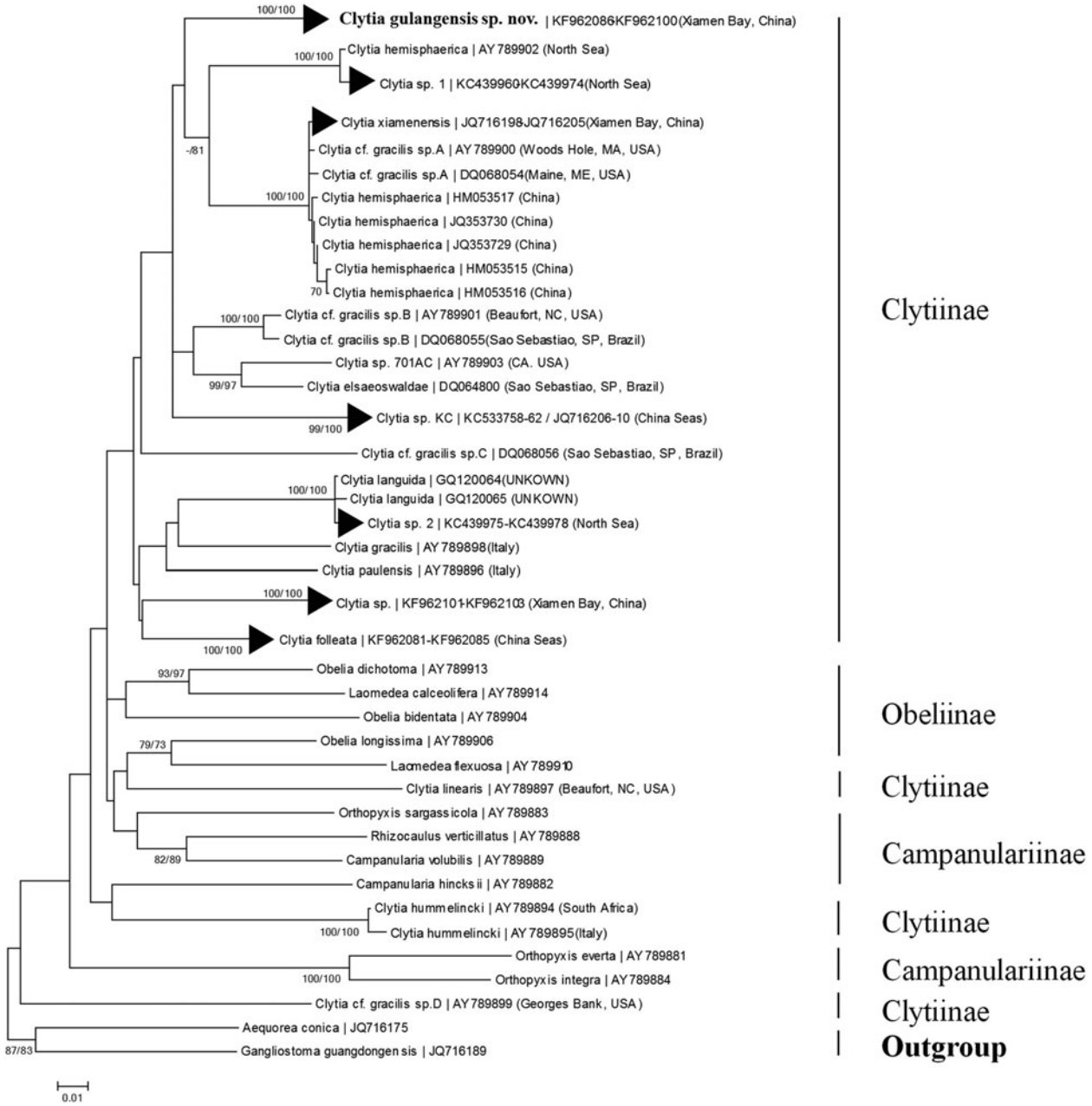

Fig. 5. Neighbour-joining clustering of Campanulariidae based on mitochondrial COI sequences. Branch support values given as bootstrap values higher than 70 obtained from maximum likelihood and neighbour-joining analyses are shown close to each branch.

from China seas, medusae of *C. hemisphaerica*, *C. languida*, *C. linearis* and *C. xiamenensis* all have equivalent number of tentacles and oval to linear gonads extending distal half of radial canals, which would show great resemblance with immature medusae of *C. gulangensis* sp. nov. as well (Huang, 2008; Huang & Lin, 2012; Zhou *et al.*, 2013). However, COI based DNA barcoding effectively separated these ambiguous individuals by adequate genetic distance and monophyletic clustering (Figure 5; Table S2), as was addressed by Laakmann & Holst (2014) to attribute unsorted morphological groups to *Clytia* and *Obelia* species. Moreover, medusae of four other species collected in the same region as the present study are also distinctly identified through COI barcoding (Figure 5; Table S2). Due to its accuracy and regardless of morphological variation, DNA barcoding would be the first choice to achieve a better understanding about *Clytia* biodiversity from field zooplankton specimens.

Taxonomic position of *C. gulangensis* sp. nov. in typical *Clytia* clade were confirmed with both 16S and 18S genes. In the present maximum likelihood phylogenetic tree based on 16S plus 18S dataset, *C. gulangensis* sp. nov. was more closely related to *C. elsaeoswaldae*, *C. xiamenensis*, and *C. hemisphaerica* than other *Clytia* species (Figure 6). While both *C. elsaeoswaldae* and *C. xiamenensis* share inclined hydrothecal cusps with *C. gulangensis* sp. nov., the former two differ from the latter in having undulated gonothecal wall and distally placed gonads. *Clytia hemisphaerica* has rounded, symmetric cusps, but its medusae have the same tentacle numbers as *C. gulangensis* sp. nov. *Clytia hemisphaerica* has also the same undulated gonothecal wall as *C. elsaeoswaldae*

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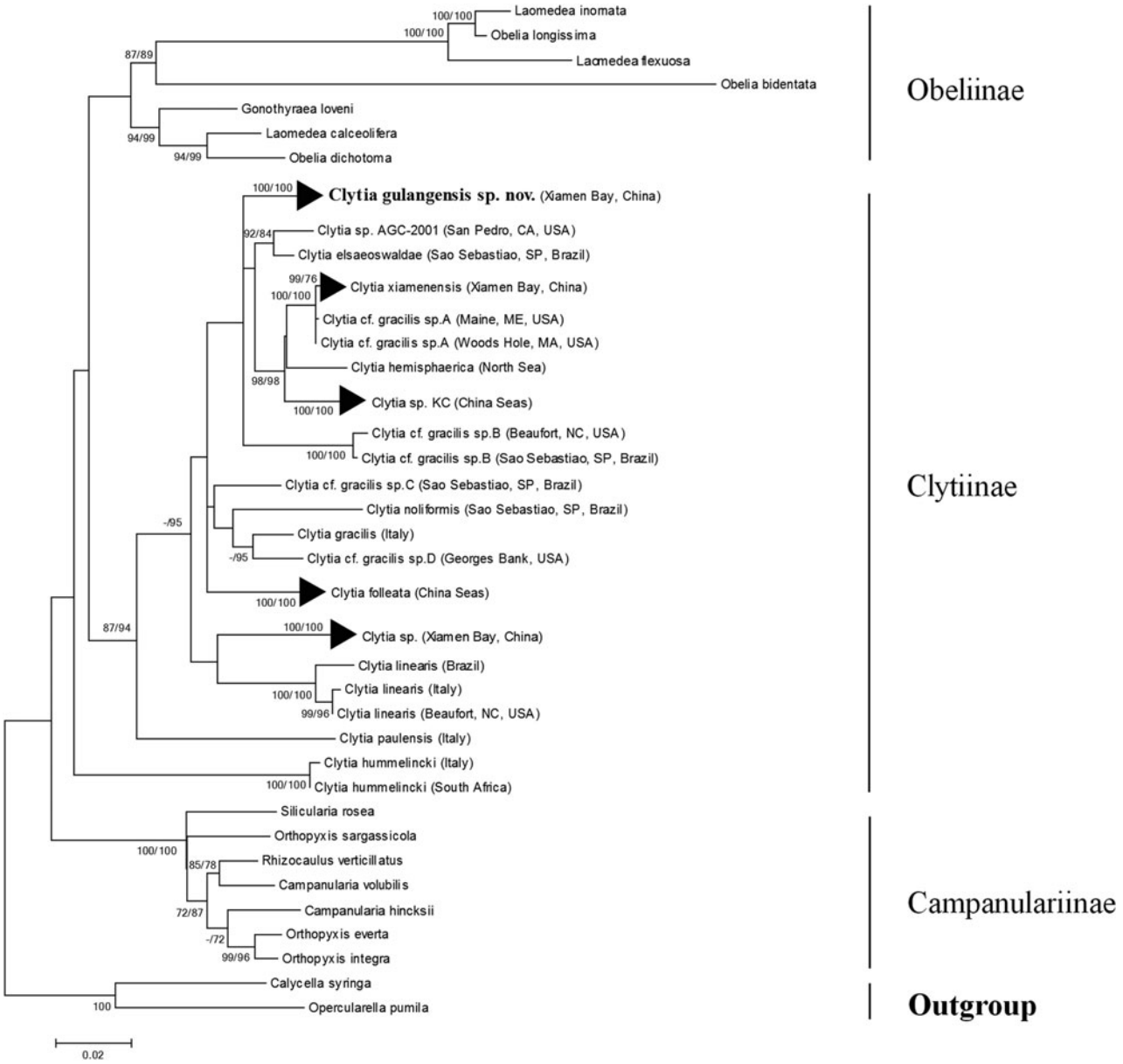

Fig. 6. Maximum likelihood phylogenetic analysis of Campanulariidae based on mitochondrial 16S and nuclear 18S rDNA. Branch support values given as bootstrap values higher than 70 obtained in maximum parsimony and maximum likelihood analyses are shown close to each branch.

and *C. xiamenensis* (Table 4). As was shown, diagnostic characters such as hydrothecal cusps, gonothecal outline, and medusae tentacles cannot fully explain the phylogenetic relationships among those species. *Clytia hummelincki*, *C. paulensis* and *C. linearis* all separated far from the *C. gulangensis* sp. nov. clade (Figure 6). Additionally, morphological characters such as the cup-like hydrotheca in *C. hummelincki* (Fraser, 1944), bibbed cusps in *C. paulensis* (Millard, 1975) and inward folds in *C. linearis* (Thornely, 1900) allow to distinguish them from *C. gulangensis* sp. nov.

Phylogenetic reconstruction revealed complicated relationships among *Clytia* species. Maximum likelihood topology of 16S plus 18S phylogenetic reconstruction placed *C. hummelincki* as a sister clade to Clytiinae plus Obeliinae lineage. Unlike other *Clytia* species, *C. hummelincki* possesses a subhydrothecal spherule and a Campanulariinae-like colony growth pattern (Fraser, 1944; Boero *et al.*, 1996; Kelmo & Attrill, 2003) and its taxonomic position was found to be basal to Campanulariinae and Clytiinae (Govindarajan *et al.*, 2006; Gravili *et al.*, 2008). The remaining *Clytia*, viz. *C. paulensis*, *C. linearis*, *C. folleata*, *C. gracilis*, *C. gulangensis* sp. nov., *C. elsaeoswaldae*, *C. hemisphaerica*, and *C. xiamenensis* formed a well supported clade (Figure 6). Species with particular morphological characters, like bibbed cusps in *C. paulensis* and inward folds in *C. linearis* do not group together, which is in accordance with the observations made in phylogenetic analysis of *C. gracilis*-like species (Lindner *et al.*, 2011).

In the context of *C. gracilis*-like species, the combined morphological and molecular evidences available tend to support these distinct genealogical lineages as separate species, rather than a single species with a strong population stratification. Firstly, though recognized by inclined hydrothecal cusps and smooth gonothecal walls, *C. elsaeoswaldae*, *C. tottoni* and *C. xiamenensis* are well morphologically described as distinct

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valid species from *C. gracilis* (Cornelius, 1995; Schuchert, 2003; Galea, 2010; Lindner *et al.*, 2011; Zhou *et al.*, 2013). In the present study, the asymmetrical pyramidal cusps, smooth embayment margin, pod-like gonothecae and medusae with linear, extended gonads and tentacle number around 30 still reveal diagnostic differences from typical *C. gracilis*. Secondly, our phylogenetic results based on COI, 16S and 18S sequences also supported the polyphyletic *C. gracilis*-like clades, which are differently related to other *Clytia* species with distinct morphological characteristics, as was stressed by Govindarajan *et al.* (2006) and Lindner *et al.* (2011). Finally, in a global molecular phylogeny of *C. gracilis*-like species, the found clades are not geographically delimited lineages. Individuals of *Clytia* cf. *gracilis* across the northern Atlantic Ocean (from Brazil and USA) showed minimal variations (e.g. *Clytia* cf. *gracilis* sp. B, Lindner *et al.*, 2011). And *C. xiamenensis*, described as a new species from the same region as the present species, has also been found in Woods Hole and Maine, USA (Zhou *et al.*, 2013) (Figures 5 and 6).

The problem of appropriate species concepts in Hydrozoa and the recognition of cryptic species vs mere sub-species level lineages has recently been brought into discussion by Schuchert (2014). Our data on *Clytia* species contribute to this ongoing debate by providing data on morphological and molecular variations in a local population of a putative cosmopolitan species complex. By adding similar data from many other populations, this will allow in future a more comprehensive view on the global level of both species diversity and phylogenetic relationships in the genus *Clytia*.

Supplementary materials and methods

The supplementary material referred to in this article can be found online at journals.cambridge.org/mbi.

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