





# Revealing hidden diversity among upside-down jellyfishes (Cnidaria: Scyphozoa: Rhizostomeae: *Cassiopea*): distinct evidence allows the change of status of a neglected variety and the description of a new species

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## ABSTRACT

Morphological variability within *Cassiopea* is well documented and has led to inaccuracies in the establishment of species boundaries in this taxon. *Cassiopea* medusae specimens from the Western Pacific (Japan and the Philippines) were analysed using multiple lines of complementary evidence, including types of cnidae, macro-morphology and molecular data. These observations lead to the recognition of two distinct species: *Cassiopea mayeri*, sp. nov. and a previously synonymised variety now raised to species level (*Cassiopea culionensis*, stat. nov.). These species can be distinguished from each other using morphological features. Herein, sexually dimorphic traits are included for the first time in the descriptions of *Cassiopea* species. Nematocyst types not previously observed in the genus are also reported. Molecular analyses, based on individual and combined markers (16S + cytochrome c oxidase I, COI), also support two distinct species; they are not sister taxa, and both are nested together within a clade of other *Cassiopea* members from the Australian and Indo-Pacific regions. Species richness is underestimated in the Western Pacific region, and integrative approaches are helpful to reveal and describe species. The systematics of *Cassiopea* is far from completely understood, but the present study represents an important further step.

<http://www.zoobank.org/References/BIA66787-009D-4465-954A-412C6878FCB4>.

**Keywords:** *Cassiopea*, DNA barcoding, jellyfish, morphology, Pacific Ocean, phylogeny, species delimitation, taxonomy.

## Introduction

Establishing species boundaries using macromorphological characters alone can be a difficult task, particularly when studying cryptic taxa (Jörger and Schrödl 2013). Modern taxonomy has become integrative by incorporating multiple lines of complementary evidence in order to provide more robust hypotheses of species delimitation (Dayrat 2005). In scyphozoan jellyfishes (phylum Cnidaria), recent taxonomic studies have incorporated evidence derived from cnidomes, statistical morphological approaches and phylogenetic hypotheses based on morphological and molecular characters (Morandini and Marques 2010; Kolbasova *et al.* 2015; Avian *et al.* 2016; Scorrano *et al.* 2016; Bayha *et al.* 2017; de Souza and Dawson 2018).

Integrative approaches are essential for detecting hidden diversity and refining the systematics of Scyphozoa (Dawson 2005a). For example, a recent study of Scyphozoa in the Tropical Eastern Pacific (TEP) integrating genetic and morphological evidence recognised 22 new species, increasing by 5-fold the known endemic scyphozoan species in the region. This confirmed that TEP (an ecoregion where scyphomedusan diversity was poorly investigated) is a biodiversity hotspot of jellyfishes (Gómez Daglio and

Dawson 2017). Thus, extensive sampling efforts coupled with integration of morphological and molecular data are essential for obtaining more reliable estimations of species richness of jellyfish.

Since the earliest Miocene, most areas in South-East Asia have been biodiversity hotspots (Becking et al. 2011; Johnson et al. 2015). Indeed, the Western Pacific Coral Triangle, a marine region that includes waters of the Philippines, Indonesia and the Solomon Islands, houses a large diversity of marine organisms, such as corals, echinoderms, fish and molluscs (Briggs 2005). Some areas of the Western Pacific also harbour diverse jellyfishes that belong to order Rhizostomeae (Mayer 1915; Kramp 1970). For example, in the Philippines, it is possible to find members of all families and 8 of 11 genera in the rhizostome suborder Kolpophorae (see Kramp 1961). This is consistent with the common view that the Philippines is the ‘epicenter of marine biodiversity,’ exhibiting the planet’s highest species richness of marine fauna (Sanciangco et al. 2013; Förderer et al. 2018; Pinheiro et al. 2019). Although multiple surveys of scyphomedusae have been conducted in the Philippines (see Mayer 1915, 1917; Light 1914, 1921), many jellyfish taxa likely remain undiscovered owing to the presence of cryptic species of jellyfish in the region (Boco and Metillo 2018).

Among members of Kolpophorae, the genus *Cassiopea* Péron & Lesueur, 1810 is known for its cryptic species (Holland et al. 2004). Identifying species of *Cassiopea* has been challenging mainly owing to the morphological variability of their medusae, which makes it difficult to distinguish their species (Mayer 1910; Gohar and Eisawy 1960; Hummelinck 1968; Morandini et al. 2017). Ten morphospecies of *Cassiopea* are currently valid (Jarms and Morandini 2019; Collins et al. 2020), and three of them occur in the Philippines: *Cassiopea andromeda* (Forskål, 1775), *Cassiopea medusa* Light, 1914 and *Cassiopea ornata* Haeckel, 1880 (Mayer 1910; Light 1914; Kramp 1961). Regarding those records, the specimens of *C. andromeda* were originally described as *Cassiopea polyoides* var. *culionensis* Light, 1914 but were later synonymised to *C. andromeda* (Stiasny 1921; Kramp 1961; but see Stiasny 1926). *Cassiopea medusa* is considered a doubtful species (Jarms and Morandini 2019). Furthermore, *C. ornata* was identified based on specimens that are not entirely similar to *C. ornata* medusae, i.e. ‘...a closely related variety of, if not identical with, *C. ornata*...’ (Mayer 1910, p. 648). Clearly, those records are uncertain, and further taxonomic studies on the genus from the Western Pacific are needed.

Reliable taxonomy of the many *Cassiopea* species is urgently needed due to the demand of documenting species under the current biodiversity crisis (Wilson 1992; Li et al. 2020) and the importance of the genus as a ‘model system’ (Ohdera et al. 2018). The genus is a favourable model system because several features of the genus are convenient in experiments, including its small genome size (~390

Mbp), its symbiotic relationship with photosynthetic dinoflagellates, ease of culturing polyps and medusae in the laboratory, and the epibenthic life habit of the jellyfish. In addition, the ephyrae and medusae are relatively large, which facilitates prompt detection of reactions to anthropogenic stressors such as coastal contaminants and ocean warming (Adachi et al. 2017; Nath et al. 2017; Ohdera et al. 2018; Medina et al. 2021). Although multiple achievements have been made using *Cassiopea xamachana* Bigelow, 1892 and *C. andromeda* as model organisms, most of the studies on *Cassiopea* are based on unidentified species (e.g. Klein et al. 2017; Rädicker et al. 2017; Aljbour et al. 2019). Since most questions in biological research mainly depend on accurate species identification (Dayrat 2005), working with unidentified *Cassiopea* hinders research on biogeography, ecology and the evolution of the jellyfish group, the detection of invasive species, and application of different species of *Cassiopea* in fields such as aquaculture, conservation and fisheries (Ohdera et al. 2018).

In this study, we determined the identity of specimens of *Cassiopea* collected in biodiversity hotspots of the Western Pacific. Our data show that these specimens belong to two species, one of which is new to science and one previously synonymised variety (now raised to species level). Thus, we increased the number of valid species of *Cassiopea* to 12. This study advances the systematics and taxonomy of an important marine group, and allows us to infer that species richness in the Western Pacific is still underestimated.

## Materials and methods

### Sample acquisition

Fifteen tissue samples for molecular analyses were collected on 11 May 2017 in Lapu-Lapu, Cebu, central Visayas, Philippines (10.285649°N, 124.000681°E). Ten additional specimens were collected in the same region (10.285967°N, 124.000750°E) on 01 March 2019; these were photographed, sub-sampled for molecular analyses and then preserved for morphological examination. In both cases, tissue samples were fixed in 96% ethyl alcohol and stored at –20°C in the Laboratory for Cnidarian Studies and Cultivation of the University of São Paulo (USP; gACM 00279–00293, 00339–00348). The specimens collected in 2019 for morphological observations were fixed and preserved in buffered 4% formaldehyde–seawater solution, and deposited in the Museu de Zoologia, University of São Paulo (MZUSP 8631-40).

Further, three specimens from the Florida Museum of Natural History, University of Florida (UF; two of which have as a primary repository the California Academy of Sciences – CASIZ) were included for morphological observations and molecular analyses: UF 009664/CASIZ 201000 and UF 009665/CASIZ 200996 from Luzon Island, Philippines, and UF 007505 from Okinawa, Japan. Tissue



**Fig. 1.** Map showing the collection sites of the studied animals (red and black circles), records from literature (green squares) and records of unidentified *Cassiopea* species from the site iNaturalist.org (lilac hexagons). Red circles represent collected specimens for this study and black ones are specimens housed in museum collections.

samples from the UF specimens are stored in the Genetic Resources Repository of the UF, and they are associated with the catalogue number of the morphological vouchers. Additionally, specimens housed in the Smithsonian National Museum of Natural History (USNM 27941), from Luzon Island, Philippines, were inspected for morphological purposes.

A distribution map was produced using SimpleMappr (D. P. Shorthouse, see <http://www.simplemappr.net>) and included records from the aforementioned specimens, literature records (Light 1914) and photographic reports of *Cassiopea* from the site iNaturalist (see <https://www.inaturalist.org>, accessed February 2020) (Fig. 1). When GPS coordinates were not available, they were estimated using Google Earth (ver. 9.124.0.1, see <https://earth.google.com/web/>).

Specimens UF 009664/CASIZ 201000 and UF 009665/CASIZ 200996 were collected under a Gratuitous Permit

(GP-0077-14) from the municipality of Calatagan (province of Batangas, Philippines).

Specimens were collected in accordance with terms and conditions of the gratuitous permit and under the supervision of the Philippines Bureau of Fisheries and Aquatic Resources (BFAR; Fisheries Regulatory and Quarantine Division) and the Philippines National Fisheries Research and Development Institute (NFRDI). The specimens from the Philippines sampled in 2017 and 2019 were collected under the permit number Fisheries Management, Regulatory and Enforcement Division (FMRED) 08190017 of the Philippine BFAR and a 'collection permit' from Lapu-Lapu City, central Philippines.

### Morphological observations and cnidome

All specimens were removed from jars and placed in a circular glass dish. Following the approach conducted by Gómez Daglio and Dawson (2017), pictures of the oral and

aboral views of the whole animals were taken, as well as close-ups of the bell margin, oral disc and oral arms. Subsequently, the specimens were removed from the dish and placed on a crystal flat surface without water. We took pictures under two types of lighting, directly exposing the specimen to an underlying light source and on a black background. We photographed the specimens using a Nikon D3300 (or a Nikon D7000) equipped with an AF Micro-Nikkor 60 mm f/2.8D macro lens (Nikon, Tokyo, Japan).

The specimens were measured with a digital calliper and the following characters were inspected: colouration, shape, size and texture of umbrella; flexibility of mesoglea; shape and number of marginal lappets between two successive rhopalia (i.e. per paramere); presence or absence of ocelli in the rhopalia, dorsal rhopaliar pit shape; subumbrellar musculature; shape and size of subgenital ostia; length and shape of oral disc; length and ramification pattern of the oral arms; abundance, distribution and shape of the oral appendages; shape of the gastrovascular cavity; when visible, number and shape of gonads; and radial canal system (when allowed, stained with a food dye diluted in tap water). The terminology of the structures follows Mayer (1910), Hummelinck (1933), Larson (1997) and Gershwin et al. (2010), and the nomenclature of the appendages follows Lindley (1832) (except for tuber-shaped). The comparative comments placed in the 'systematics remarks' are based on the comparison of our material and the original descriptions of other species of *Cassiopea* from the region. The general outline of the descriptions follows Morandini and Marques (2010).

A Nikon SMZ1000 stereomicroscope with an attached DS-Ri1 digital camera was used to observe the sense organs, determine the sex and examine the oral appendages under higher magnification. Undischarged nematocyst capsules were identified and measured (when possible 30 of each type) from squash preparations made of formaldehyde-preserved pieces of tissue from the oral surface of the oral arms. Preparations were inspected at 1000× using a Nikon Eclipse 80i optical microscope with a DS-Ri1 digital camera. We followed the classification of Heins et al. (2015) and Morandini and Marques (2010).

When necessary, we corrected image parameters using GIMP (ver. 2.8, see <https://www.gimp.org>), and we edited the figures using Inkscape (ver. 0.91, see <https://inkscape.org/>).

## Molecular protocols and data analyses

### DNA extraction, amplification and sequencing

DNA was isolated using an ammonium acetate DNA extraction procedure (Fetzner 1999). From each specimen, we amplified a ~615-bp fragment of the mitochondrial ribosomal gene *16S* rRNA (hereafter *16S*) and a 650-bp fragment of the mitochondrial protein-encoding gene cytochrome *c* oxidase subunit *I* (hereafter *COI*). *COI* was amplified using the same primers and PCR conditions used in

Gamero-Mora et al. (2019) or using 'LCO1490-JJ2' (5'-CHACWAAAYCAYAARGAYATYGG-3') and 'HCO2198-JJ2' (5'-ANACTTCNGGRTGNCCAAARAATCA-3') under a touch-down and step-up PCR protocol (Astrin et al. 2016). *16S* was amplified using the primers 'C&B1' (5'-TCGACTGTTTAC CAAAAACATAGC-3') and 'C&B2' (5'-ACGGAATGAACTCAAA TCATGTAAG-3') (Cunningham and Buss 1993); thermal cycling conditions were 3 min at 95°C for initial denaturation, followed by 35 cycles of amplification (denaturation at 95°C for 35 s, annealing at 49°C for 40 s and extension at 72°C for 50 s) and a final extension for 7 min at 72°C. The amplicons were purified either using Agencourt AmPure XP (Beckman Coulter, Brea, CA, USA) or ExoSapIT (Affymetrix, Santa Clara, CA, USA), and subsequently used in cycle sequencing reactions together with the reagents of the Big Dye Terminator Cycle Sequencing Kit (ver. 3.1, Applied Biosystems, Foster City, CA, USA) and the primers from PCR. Cycle sequencing products were either cleaned and precipitated using 3-M sodium acetate and ethyl alcohol or purified with Sephadex G-50 (Sigma-Aldrich, Buchs, Switzerland), and sequenced bidirectionally on an ABI 3730xl DNA Analyzer (Applied Biosystems).

### Phylogenetic reconstructions and other molecular analyses

Sequenced chromatograms were assembled, inspected and trimmed using Geneious (ver. 6.1.8, Biomatters, Auckland, New Zealand).

The newly generated sequences were deposited in GenBank (MW160911–MW160937, MW164859–MW164886) and analysed, under a phylogenetic approach, together with sequences of *Cassiopea* species (*Cassiopea frondosa* (Pallas, 1774), *C. andromeda*, *C. ornata*, *C. xamachana* and *Cassiopea* sp. 1–6), *Mastigias papua* (Lesson, 1830) and *Versuriga anadyomene* (Maas, 1903) available from GenBank (Table 1, Supplementary Table S1, S2).

*16S* sequences were aligned using the E-INS-i method (command line: mafft – genafpair – maxiterate 1000) and *COI* sequences using the L-INS-i (command line: mafft – localpair – maxiterate 1000) method, in both cases using MAFFT (ver. 7.271, see <https://mafft.cbrc.jp/alignment/software/>; Katoh and Standley 2013). Alignments were trimmed at selected regions based on options for a less-stringent selection using Gblocks (ver. 0.91b, see <http://molevol.cmima.csic.es/castresana/Gblocks.html>; Castresana 2000; Talavera and Castresana 2007).

Phylogenetic analyses were run using maximum likelihood as the optimality criterion for individual markers (*16S*, *COI*) and the combined dataset (*16S* + *COI*). The optimal partition scheme and substitution models were selected with ModelFinder (subset 1: *16S*, TIM2+F+G4; subset 2: *COI* codon 1, TIM+F+I+G4; subset 3: *COI* codon 2, K3Pu+F+I; subset 4: *COI* codon 3, K3Pu+F+I+G4) (Kalyaanamoorthy et al. 2017). The phylogenetic analyses were conducted using IQ-TREE multicore (ver. 1.6.10,

**Table 1.** List of sequences used in this study of *Cassiopea* species.

Marker	Species (this study)	Voucher code	GenBank	Collection locality	Source
16S	<i>Cassiopea andromeda</i>	M0D006024R	<a href="#">KY610609</a>	Isla San Jose, Baja California Sur, Mexico	<a href="#">Gómez Daglio and Dawson 2017</a>
		N/A	<a href="#">JN700934</a>	Tiahura, Moorea, French Polynesia	<a href="#">Kayal et al. 2013</a>
	<i>C. culionensis</i> , stat. nov.	MZUSP 8633, gACM00341	<a href="#">MWI64879</a>	Lapu-Lapu, City of Cebu, Philippines	This study
		gACM00285	<a href="#">MWI64869</a>	Lapu-Lapu, City of Cebu, Philippines	This study
		MZUSP 8640, gACM00348	<a href="#">MWI64886</a>	Lapu-Lapu, City of Cebu, Philippines	This study
	<i>C. frondosa</i>	M0D014623K	<a href="#">KY610617</a>	Key West, Florida, USA	<a href="#">Gómez Daglio and Dawson 2017</a>
	<i>C. mayeri</i> , sp. nov.	FLMNH 007505	<a href="#">MWI64859</a>	Ryukyu Islands, Okinawa, Japan	This study
		gACM00280	<a href="#">MWI64863</a>	Lapu-Lapu, City of Cebu, Philippines	This study
		MZUSP 8631, gACM00339	<a href="#">MWI64864</a>	Lapu-Lapu, City of Cebu, Philippines	This study
		FLMNH 009664/CASIZ 201000	<a href="#">MWI64865</a>	Calatagan, Luzon Island, Philippines	This study
	<i>C. ornata</i>	FLMNH 009665/CASIZ 200996	<a href="#">MWI64866</a>	Calatagan, Luzon Island, Philippines	This study
		N/A	<a href="#">AB720918</a>	?Kamo Aquarium, Japan	R. O. Gotoh, C. Ito, S. Mochizuki, and N. Hanzawa, unpubl. data
	<i>C. xamachana</i>	M0D002666N	<a href="#">KY610616</a>	Koror, Palau	<a href="#">Gómez Daglio and Dawson 2017</a>
		M0D021381I	<a href="#">KY610614</a>	Bahia Delfines, Bocas del Toro, Panama	<a href="#">Gómez Daglio and Dawson 2017</a>
		N/A	<a href="#">JN700936</a>	Bocas del Toro, Panama	<a href="#">Kayal et al. 2013</a>
	<i>Cassiopea</i> sp. 4	M0D018638V	<a href="#">MZ366351</a>	Ongael Lake, Koror State, Palau	<a href="#">Gamero-Mora et al. in prep</a>
<i>Mastigias papua</i>	M0D006000T	<a href="#">KY610621</a>	Ongael Lake, Koror State, Palau	<a href="#">Gómez Daglio and Dawson 2017</a>	
<i>Versuriga anadyomene</i>	N/A	<a href="#">KX904852</a>	Beibu Gulf, South China Sea	<a href="#">Sun et al. 2019</a>	
COI	<i>C. andromeda</i>	M0D006024R	<a href="#">KY610551</a>	Isla San Jose, Baja California Sur, Mexico	<a href="#">Gómez Daglio and Dawson 2017</a>
		N/A	<a href="#">JN700934</a>	Tiahura, Moorea, French Polynesia	<a href="#">Kayal et al. 2013</a>
	<i>C. culionensis</i> , stat. nov.	MZUSP 8633, gACM00341	<a href="#">MWI60923</a>	Lapu-Lapu, City of Cebu, Philippines	This study
		gACM00285	<a href="#">MWI60913</a>	Lapu-Lapu, City of Cebu, Philippines	This study
		MZUSP 8640, gACM00348	<a href="#">MWI60930</a>	Lapu-Lapu, City of Cebu, Philippines	This study
	<i>C. frondosa</i>	N/A	<a href="#">KF683387</a>	Monterey Bay Aquarium, California, USA	<a href="#">Mellas et al. 2014</a>
		M0D021384L	<a href="#">KY610560</a>	Bahia Delfines, Bocas del Toro, Panama	<a href="#">Gómez Daglio and Dawson 2017</a>
		CFFK1	<a href="#">AY319467</a>	Key Largo Florida, USA	<a href="#">Holland et al. 2004</a>

(Continued on next page)

**Table 1.** (Continued)

Marker	Species (this study)	Voucher code	GenBank	Collection locality	Source
	<i>C. mayeri</i> , sp. nov.	FLMNH 007505	<b>MW160931</b>	Ryukyu Islands, Okinawa, Japan	This study
		gACM00280	<b>MW160934</b>	Lapu-Lapu, City of Cebu, Philippines	This study
		MZUSP 8631, gACM00339	<b>MW160935</b>	Lapu-Lapu, City of Cebu, Philippines	This study
		FLMNH 009664/CASIZ 201000	<b>MW160936</b>	Calatagan, Luzon Island, Philippines	This study
		FLMNH 009665/CASIZ 200996	<b>MW160937</b>	Calatagan, Luzon Island, Philippines	This study
		N/A	AB563739	Izu Chuo Aqua Trading Co., Ltd, ?Japan	Ojimi and Hidaka 2010
		N/A	AB563740	Enoshima Aquarium, ?Japan	Ojimi and Hidaka 2010
	<i>C. ornata</i>	CAKKII	<u>AY319472</u>	Kakaban, Kalimantan, Indonesia	Holland et al. 2004
		N/A	<u>AY319473</u>	Kakaban, Kalimantan, Indonesia	Holland et al. 2004
	<i>C. xamachana</i>	M0D021381I	<u>KY610559</u>	Bahia Delfines, Bocas del Toro, Panama	Gómez Daglio and Dawson 2017
		N/A	<u>JN700936</u>	Bocas del Toro, Panama	Kayal et al. 2013
	<i>Cassiopea</i> sp. 1	CAPDNSWI	<u>AY319471</u>	Port Douglas, Queensland, Australia	Holland et al. 2004
	<i>Cassiopea</i> sp. 2	M0D006326H	<u>MF742198</u>	Observation Point, Papua New Guinea	Abboud et al. 2018
		M0D006327I	<u>MF742199</u>	Observation Point, Papua New Guinea	Abboud et al. 2018
	<i>Cassiopea</i> sp. 3	CAWC I	<u>AY331594</u>	Windward O'ahu, Hawaii, USA	Holland et al. 2004
		CAKRI	<u>AY331595</u>	Windward O'ahu, Hawaii, USA	Holland et al. 2004
	<i>Cassiopea</i> sp. 4	N/A	<u>LC198739</u>	Milky Way Lake I, Palau	Arai et al. 2017
		N/A	<u>LC198740</u>	Ongael Lake, Koror State, Palau	Arai et al. 2017
	<i>Cassiopea</i> sp. 6	N/A	<u>LC198754</u>	NGE Lake I Lagoon, Palau	Arai et al. 2017
		N/A	<u>LC198763</u>	Palau	Arai et al. 2017
	<i>Mastigias papua</i>	M0D015702X	<u>KU901434</u>	Mekeald Lake, Palau	Swift et al. 2016
	<i>Versuriga anadyomene</i>	N/A	<u>KX904853</u>	Beibu Gulf, South China Sea	Sun et al. 2019

Note: GenBank accession numbers of sequences obtained in this study are in bold. Underlined GenBank accession numbers indicate that these sequences were used in the combined-marker analysis. FLMNH, Florida Museum of Natural History; gACM, Laboratory for Cnidarian Studies and Cultivation of the University of São Paulo; MZUSP, Museu de Zoologia, University of São Paulo; N/A, not applicable.

see <http://www.iqtree.org/>; Nguyen *et al.* 2015). Clade stability was assessed by two parametric (approximate likelihood ratio test (aLRT) and a Bayesian-like transformation of aLRT (aBAYES)) and two non-parametric methods (standard bootstrap, Shimodaira–Hasegawa (SH)-aLRT; 1000 replicates). The final alignments and trees were deposited in Figshare (see <https://doi.org/10.6084/m9.figshare.16528203>).

Uncorrected pairwise *p*-distances were calculated in Geneious (ver. 6.1.8) and visualised as heatmaps using RStudio (ver. 1.4.1106, see <https://www.rstudio.com/>).

We used PopART (ver. 1.7, see <http://popart.otago.ac.nz/index.shtml>; Leigh and Bryant 2015) to generate Templeton–Crandall–Sing (TCS) haplotype networks for the 16S and COI alignments (Supplementary Table S3). Haplotype networks were edited in Inkscape (ver. 0.91).

## Results

The inspected specimens belong to two different species. *Cassiopea mayeri*, sp. nov., here described, and *C. polypoides* var. *culionensis* (described originally in 1914 by Light and synonymised with *C. andromeda* in 1921 by Stiasny; but see Stiasny 1926 and Kramp 1961), which we now raise to species level and redescribe as *Cassiopea culionensis*, stat. nov. Our species hypotheses are supported by morphological evidence and the two molecular markers used: COI, which has been already used with success in identifying cryptic species within *Cassiopea* (Holland *et al.* 2004); and 16S, which is used here for the first time for reconstructing the phylogeny of the genus.

### Genetic divergence, phylogenetic placement and haplotype networks

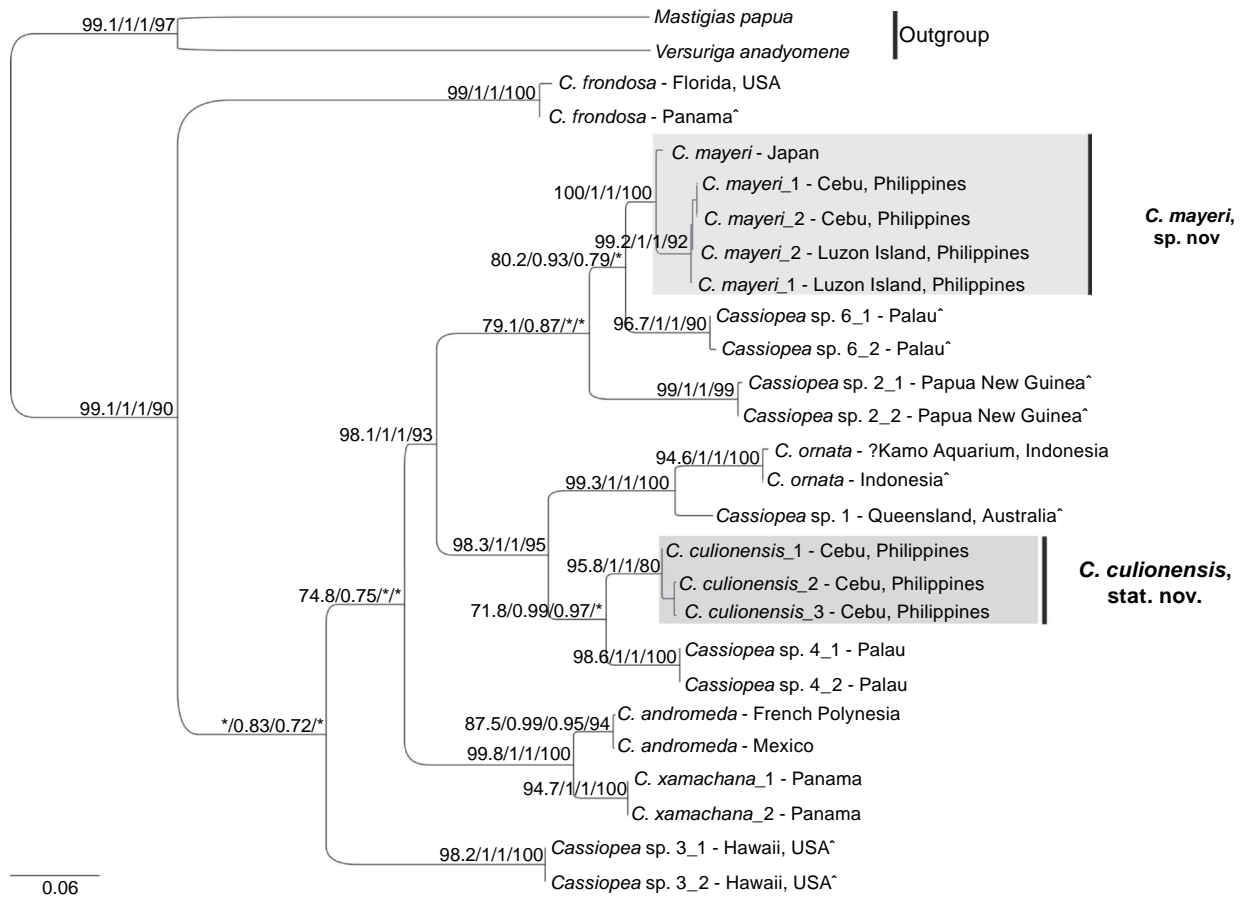
The *p*-distance (%) between *C. mayeri*, sp. nov. and *C. culionensis*, stat. nov. is 10.1–12.9 for 16S (Supplementary Fig. S1a) and 13.6–14.2 for COI (Supplementary Fig. S2a). The COI value is higher than the minimum value found between valid (*C. andromeda* v. *C. xamachana* = ~7.3%) and valid-candidate *Cassiopea* species (*C. ornata* v. *Cassiopea* sp. 1 = ~9.4%). The result of our combined phylogenetic analysis is presented in Fig. 2 (Table 1, Supplementary Table S1). It supports our morphological results showing that the sequenced individuals can be hypothesised as two distinct taxa, which were recovered in two different monophyletic groups with high clade stability values. Each group is placed in different positions in the phylogeny of *Cassiopea*, i.e. they are not sister taxa. Low clade stability values were found in deeper nodes of the phylogeny (but see Supplementary Fig. S3, Supplementary Table S2 for the analysis without missing data). Regarding the individual markers analyses (Table 1, Supplementary Fig. S1b, S2b), both markers support the results of the combined phylogenetic analysis (Fig. 2), but with some

differences in the internal branching (but note that we did not include all putative species in the analyses).

The analysis of COI sequences failed to confirm the monophyly of *C. culionensis*, stat. nov. Likewise, it showed that *C. culionensis*, stat. nov. does not belong to any already proposed candidate species (i.e. *Cassiopea* sp. 1–6) (Supplementary Fig. S2b). However, the analysis revealed that one sequence retrieved from GenBank (KF683387 – Mellas *et al.* 2014, pp. 39–40), obtained from an individual referred to as *C. ornata* and raised in the Monterey Bay Aquarium, belongs to *C. culionensis*, stat. nov. In the concatenated tree (Fig. 2), *C. culionensis*, stat. nov. is recovered as the sister group of *Cassiopea* sp. 4, which together are the sister group of *C. ornata* + *Cassiopea* sp. 1.

We linked *Cassiopea* sp. 5 with the specimens classified here as *C. mayeri*, sp. nov. *Cassiopea* sp. 5 was proposed by Arai *et al.* (2017) to name COI sequences obtained from individuals cultured in the Enoshima Aquarium and from Izu Chuo Aqua Trading Co., Ltd, Japan (AB563739–40 in Ojimi and Hidaka 2010). The sampling site of their original cultures is unknown, but they grouped with our specimen from Okinawa, Japan (Supplementary Fig. S2b). Thus, *C. mayeri*, sp. nov. is composed of two main clades, one of them including specimens from Japan and another with specimens from the Philippines. Based on morphological similarity (even with recognised variability, see variation section) and tree topology, we describe both main clades as *C. mayeri*, sp. nov. The *p*-distance (%) between individuals from Japan and the Philippines is 1.4–1.8 for 16S (Supplementary Fig. S1a) and 3.2–4.2 for COI (Supplementary Fig. S2a, b). In the combined tree (Fig. 2), *C. mayeri*, sp. nov. is recovered as the sister group of *Cassiopea* sp. 6, and *Cassiopea* sp. 2 is the closest species related to them.

Haplotype networks of the marker 16S revealed three haplotypes of *C. culionensis*, stat. nov. segregated per a maximum of five mutational steps (one haplotype corresponded to a single individual and the other two were shared) and five haplotypes of *C. mayeri*, sp. nov. segregated per a maximum of seven mutational steps (three haplotypes corresponded to single individuals and the other two were shared). There was one 16S haplotype shared among Luzon and Central Visayas (Lapu-Lapu) (Fig. 3a). Conversely, haplotype networks of the marker COI showed seven haplotypes of *C. culionensis*, stat. nov. segregated per a maximum of two mutational steps (three haplotypes corresponded to single individuals and the other four were shared), and five haplotypes of *C. mayeri*, sp. nov. segregated per a maximum of 19 mutational steps (two haplotypes corresponded to single individuals and the other three were shared). In the case of *C. mayeri*, sp. nov., the maximum number of mutational steps (seven for 16S and 19 for COI) was found between populations from the Philippines and Japan (Fig. 3b). *Cassiopea mayeri*, sp. nov. was separated by 50 (16S) and 71 (COI) mutational steps from *C. culionensis*, stat. nov.



**Fig. 2.** Maximum likelihood tree based on mitochondrial ribosomal gene *16S* rRNA and mitochondrial protein-encoding gene cytochrome *c* oxidase I (*COI*) data ( $-\ln L$  4852.50). Grey rectangles indicate sequences obtained during this study. Clade stability values are shown on branches (as in figure order: Shimodaira–Hasegawa (SH)-approximate likelihood ratio test (aLRT) (%), parametric aLRT, aBayes, bootstrap values (%); asterisks (\*) indicate less than 0.7, 70). For taxa names and other information see Table 1, Supplementary Table S1. Circumflexes (^) represent taxa with only *COI* data.

## Taxonomy

Order **RHIZOSTOMEAE** Cuvier, 1800

Suborder **KOLPOPHORAE** Stiasny, 1920

Family **CASSIOPEIDAE** Tilesius, 1831

Genus ***Cassiopea*** Péron & Lesueur, 1810

***Cassiopea culionensis*** Light, 1914, stat. nov.

(Fig. 1, 4–8)

*Cassiopea polypoides* var. *culionensis* Light, 1914, pp. 201–203 (original description) (Culion Bay, Philippines). – Stiasny (1926), p. 245 (*C. polypoides* var. *culionensis* is re-validated).

*Cassiopea andromeda* (partim.) – Stiasny (1921), p. 69 (*C. polypoides* var. *culionensis* is a synonym of *C. andromeda*); Kramp (1961), p. 349 (mention *C. polypoides* var. *culionensis* as a synonym of *C. andromeda*);

Jarms and Morandini (2019), p. 484 (mention *C. polypoides* var. *culionensis* as a synonym of *C. andromeda*).

*Cassiopea ornata* (partim.) – Mellas et al. (2014), pp. 39–40 (GenBank accession number: KF683387; study of the variation in symbiont uptake in early stages of *Cassiopea* development).

*Type locality*: Culion Bay, Philippines.

## Material examined

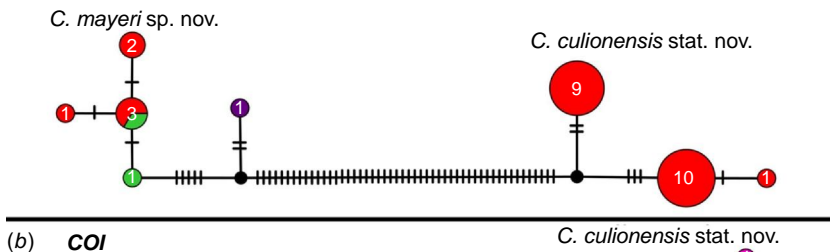
*Neotype specimen*. Specimen C. 2420, Zoological Collection, University of the Philippines was most likely lost during World War II; a neotype is designated herein (Museu de Zoologia, University of São Paulo; MZUSP 8634).

*Neotype* (MZUSP 8634). One adult male, 9.3 cm in bell diameter, from Lapu-Lapu, Cebu, Philippines, 10.285967°N 124.000750°E; coll. S. M. Geson III, hand captured, 01.iii.2019, 4% formaldehyde solution in seawater; DNA subsample – ethyl alcohol preserved – voucher ID gACM00342.

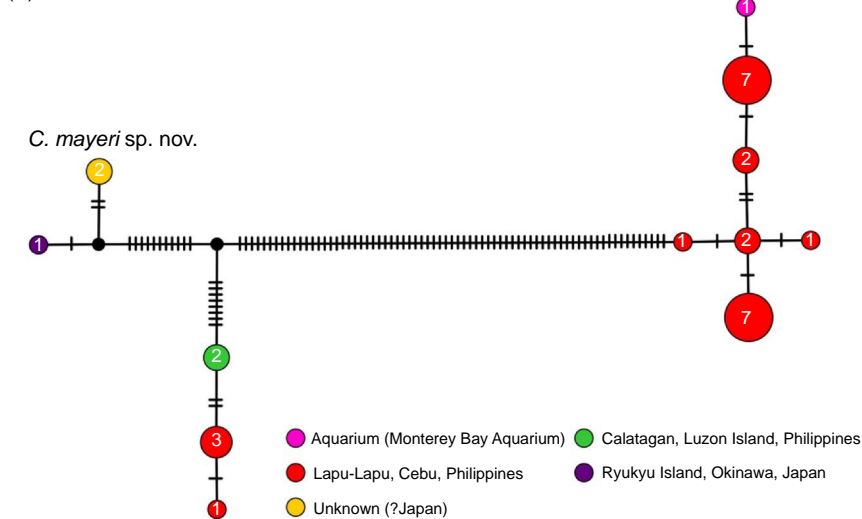
*Other specimens examined*. MZUSP 8632, one adult female, 9.4 cm in bell diameter, same data as neotype, DNA voucher ID: gACM00340. MZUSP 8633, one adult female, 11.3 cm in bell diameter, same data as neotype, DNA voucher ID: gACM00341. MZUSP 8635, one adult



## (a) 16S



## (b) COI



**Fig. 3.** Haplotype networks for specimens of *Cassiopea mayeri*, sp. nov. and *Cassiopea culionensis*, stat. nov. based on mitochondrial ribosomal gene 16S rRNA and mitochondrial protein-encoding gene cytochrome c oxidase I (COI) sequences. Sizes of the circles are proportional to haplotype frequency, and bars indicate the number of mutations between two haplotypes. Black circles are hypothetical nodes; haplotype colours match the geographic origin of the samples. (a) Haplotype networks derived from 16S sequences of *C. mayeri*, sp. nov. (eight specimens, 569 bp) and *C. culionensis*, stat. nov. (20 specimens, 569 bp). (b) Haplotype networks derived from COI sequences of *C. mayeri*, sp. nov. (9 specimens, 582 bp) and *C. culionensis*, stat. nov. (21 specimens, 582 bp). Supplementary Table S3 contains further details on sequences used to construct the haplotype networks.

female, 8.6 cm in bell diameter, same data as neotype, DNA voucher ID: gACM00343. MZUSP 8636, one adult male, 9.9 cm in bell diameter, same data as neotype, DNA voucher ID: gACM00344. MZUSP 8637, one adult female, 9.3 cm in bell diameter, same data as neotype, DNA voucher ID: gACM00345. MZUSP 8638, one adult female, 8.8 cm in bell diameter, same data as neotype, DNA voucher ID: gACM00346. MZUSP 8639, one adult female, 8.7 cm in bell diameter, same data as neotype, DNA voucher ID: gACM00347. MZUSP 8640, one adult female, 11.3 cm in bell diameter, same data as neotype, DNA voucher ID: gACM00348.

### Diagnosis (but see also Discussion)

Oral arms with small parabolic or orbicular appendages throughout; females with a central flattened appendage surrounded by a whorl of numerous similar appendages of different sizes often folded, and males with parabolic or only small linear appendages in the central disc; trapezoid rhopalial pit; five marginal lappets per paramere.

### Neotype specimen description (MZUSP 8634)

*Formaldehyde preserved.* Specimen in good condition, 9.2 cm in bell diameter.

*Exumbrella* (Fig. 4a, b). Transparent and slightly bulging in central region, slightly depressed around bulging region, gradually rising to exumbrellar raised ring (*ringwulst*); smooth, except for *ringwulst*.

*Mesoglea.* Flexible, thinner at edge.

*Marginal lappets* (Fig. 5e). Five per paramere (three velar flanked by two ocular), blunt, central lappet the widest.

*Rhopalia* (Fig. 4c, d, 5d). Seventeen; from exumbrellar side, each sense organ in a trapezoid-shaped rhopalial pit; ocelli not observed (maybe due to preservation); from subumbrellar side, sides of the rhopalial pits partially covered by folding of two ocular lappets.

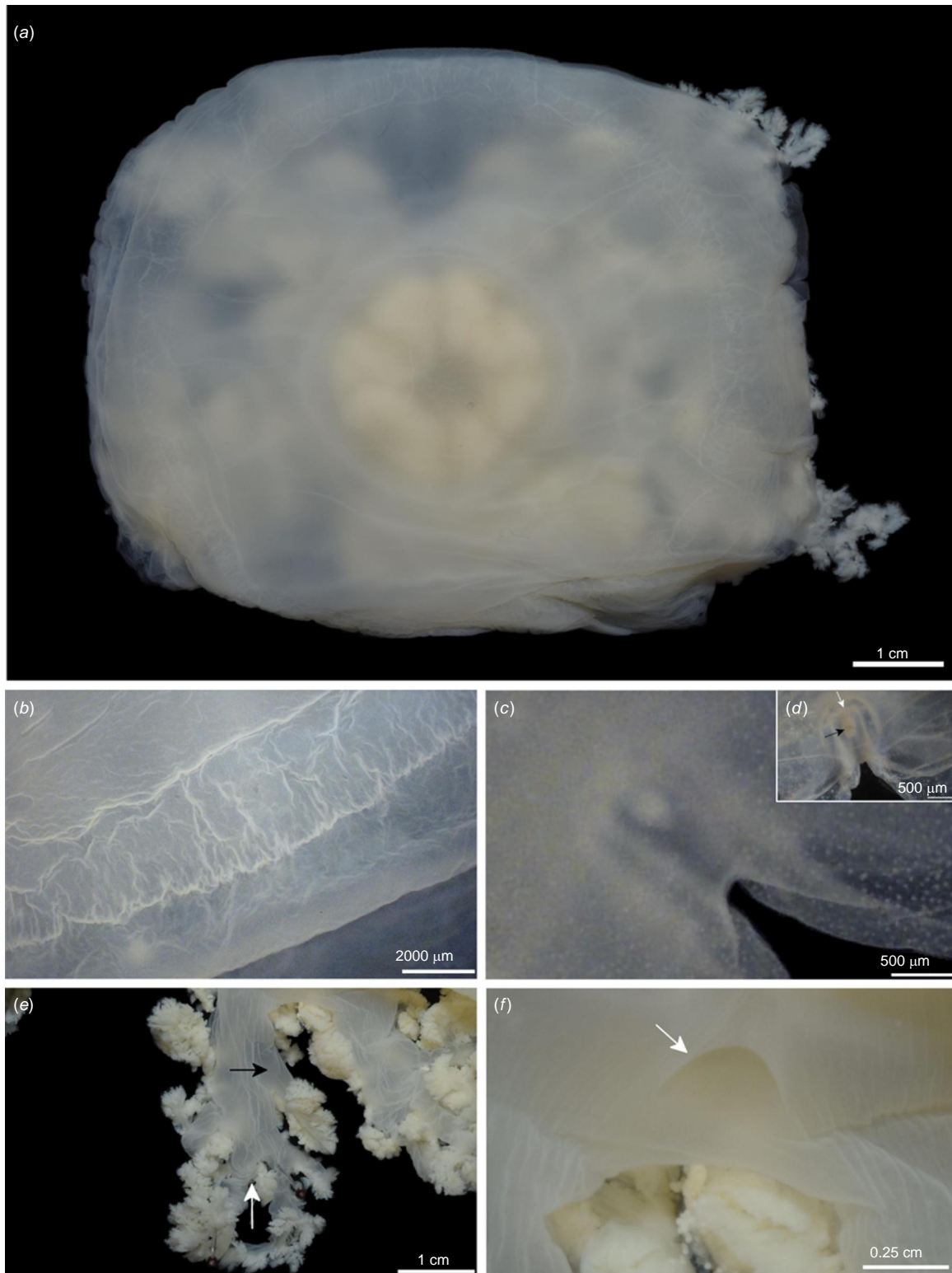
*Subumbrellar musculature.* Repeated pattern of fine U- or V-shaped muscle bands forming arcades.

*Subgenital ostia* (Fig. 4f). Four small, interradial, U-shaped, 0.28 cm wide and 0.22 cm high.

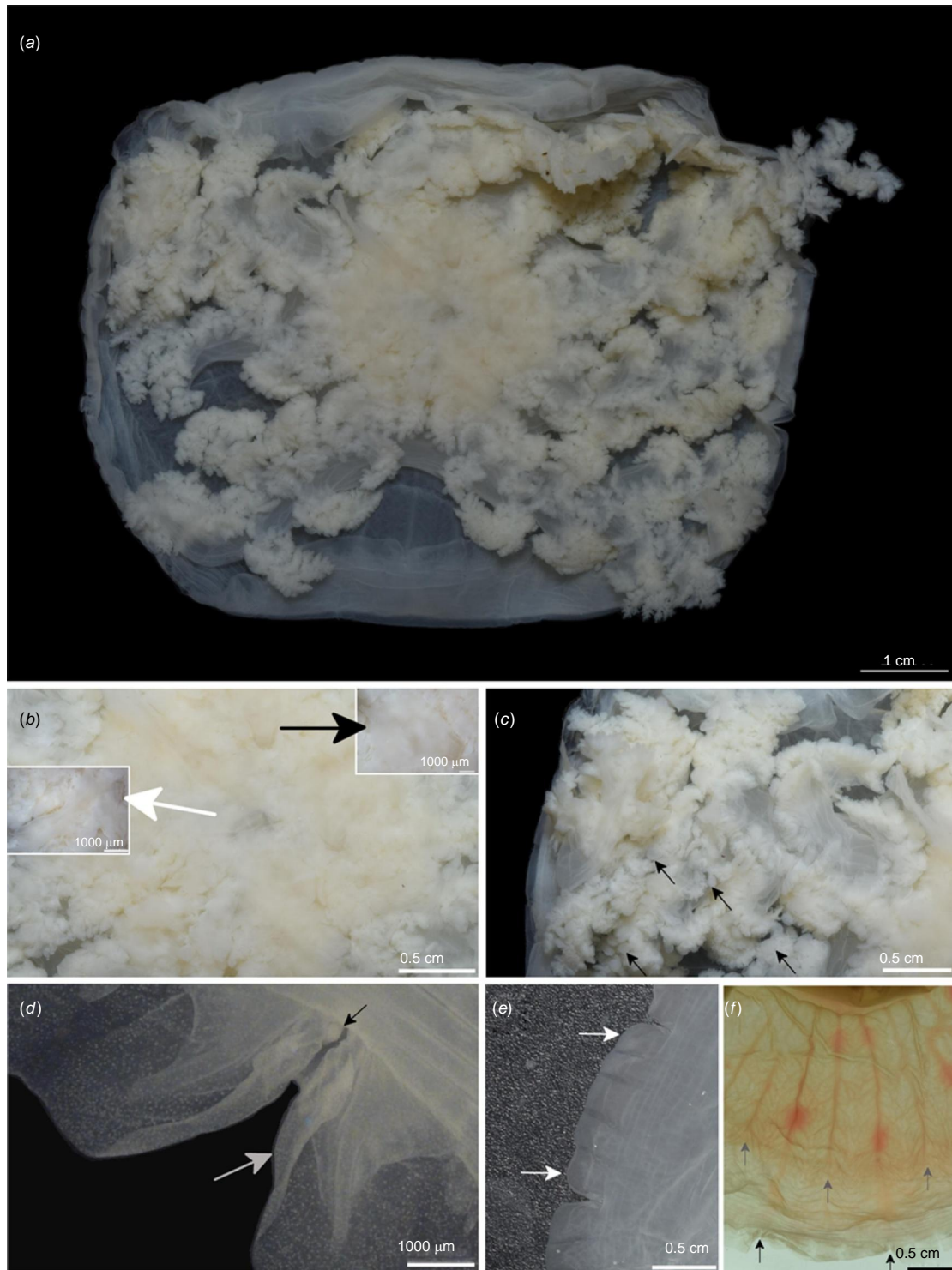
*Oral arm disc* (Fig. 5a). Flat, exceeds one-third of bell diameter, 3.26 cm wide.

*Oral arms* (Fig. 4e, 5a). Eight adradial mouth arms (four pairs) arise from the oral disc at the centre of subumbrella; variable in size, but usually exceed length of one-third of bell diameter, 3.66 cm in length, some extend beyond bell margin. Approximately 3–5 lateral branches alternate in position along central trunk of the arm; central trunk ends in a bifurcation that starts before last quarter of main trunk.

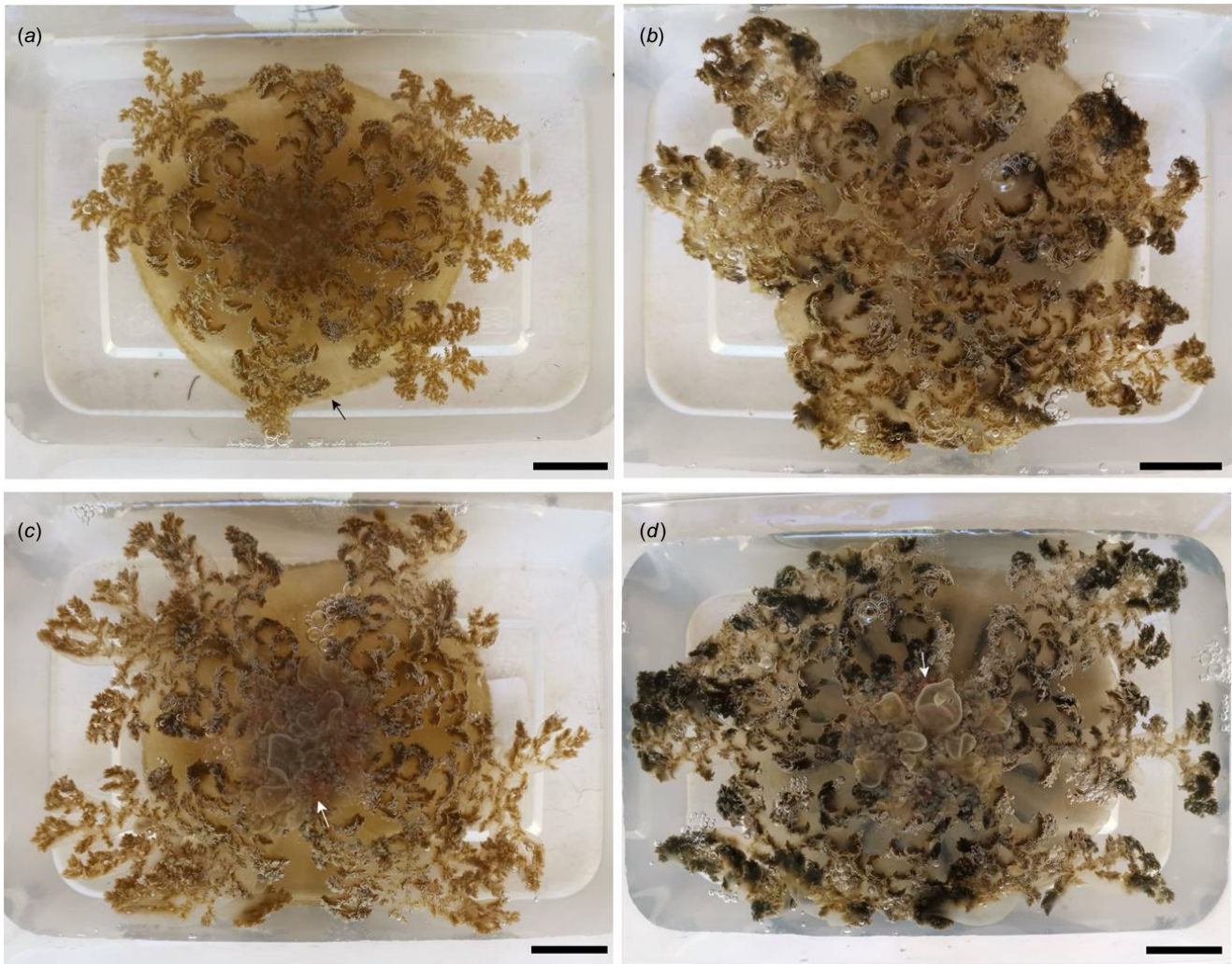
*Oral appendages* (Fig. 5a–c). Oral disc with one long central appendage, oblong at base, linear towards end, 1.8 cm long, and 1–2 parabolic-shaped appendages in axil of each pair of oral arms, 6 mm long; each arm with



**Fig. 4.** *Cassiopea culionensis*, stat. nov., aboral views of the formaldehyde-preserved male neotype (MZUSP 8634). (a) Aboral view. (b) Exumbrellar raised ring (*ringwulst*). (c) Exumbrellar view of a rhopalium and rhopalial pit. (d) Exumbrellar view of a rhopalium with ocellum and rhopalial pit. Black arrow indicates the rhopalium with ocellum (MZUSP 8637), white arrow indicates the base of a trapezoid-shaped rhopalial pit. (e) Branching pattern of the oral arm. Black arrow indicates a lateral branch, white arrow indicates the beginning of the terminal bifurcation. (f) Subgenital ostium (white arrow).



**Fig. 5.** *Cassiopea culionensis*, stat. nov., oral views of the formalin preserved male neotype (MZUSP 8634). (a) Oral view. (b) Detail of the appendages at the oral disc. Black arrow indicates a parabolic-shaped appendage, white arrow indicates an appendage oblong at the base and linear towards the end. (c) Appendages of the oral arm. Black arrows indicate orbicular or parabolic appendages. (d) Rhopalium and ocular lappets. Black arrow indicates a rhopalium, grey arrow indicates the folding of an ocular lappet covering the sides of the rhopaliar pit. (e) Subumbrellar view of the bell margin showing a paramere. White arrows indicate ocular lappets. (f) Canal system showing some of the rhopaliar and inter-rhopaliar canals and anastomoses. Black arrows indicate rhopaliar canals, grey arrows indicate inter-rhopaliar canals.



**Fig. 6.** *Cassiopea culionensis*, stat. nov., oral view of live specimens. (a) Male neotype (MZUSP 8634), umbrella in amber tone. (b) Male specimen MZUSP 8636, umbrella in ivory tone. (c) Female specimen MZUSP 8632, umbrella in amber tone. (d) Female specimen MZUSP 8633, umbrella in ivory tone. Black arrow indicates whitish marks in the exumbrella, white arrows indicate regions with a cherry umbrella colour. Note in all cases the colours of the oral groove. Scale bars: 2 cm.

parabolic or orbicular appendages especially abundant in oral disc and distal region of oral arms, 1 mm long.

**Gastrovascular cavity** (Fig. 4a). Circular or quadrangular, 3.05 cm in diameter.

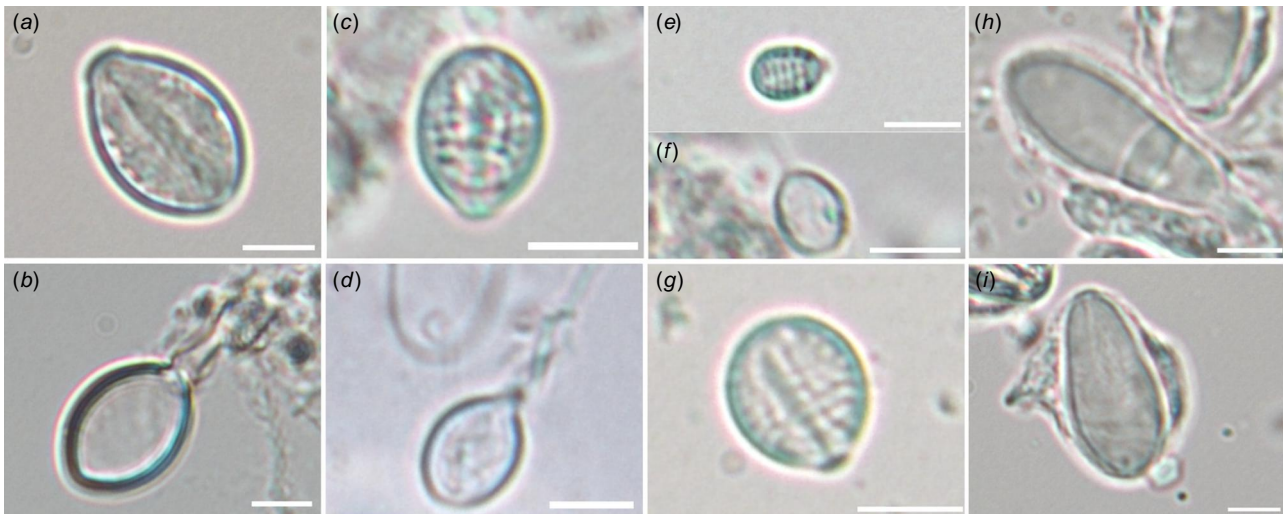
**Gonads** (Fig. 4a). Four gonads are spread out forming a cross within stomach diameter.

**Canal system** (Fig. 5f). A main canal extends from each of eight oral arms into gastrovascular cavity, where 34 radial canals arise and go along the subumbrella, communicating by a network of anastomosing vessels; 17 radial canals end in a rhopalium (rhopalial canals) and 17 are intermediate in position (inter-rhopalial canals).

**Colour in 4% formaldehyde** (Fig. 4a, 5a). Translucent whitish umbrella, oral arms, central disc and appendages; creamish-yellow gonads and ventral surface of oral arms.

**Colour in life** (Fig 6a–d). Yellowish-amber umbrella, amber-brownish ventral surface of oral arms (i.e. oral groove), with whitish-amber appendages. Exumbrellar colour not documented; however, whitish marks were observed in the specimen when alive, which faded after fixation.

**Nematocysts** (for nematocysts identifications see Fig. 7). Oral appendages with holotrichous O-isorhizas ( $n = 3$ ;  $5.27\text{--}6.74 \times 4.77\text{--}5.53 \mu\text{m}$  (mean =  $5.85 \times 5.09 \mu\text{m}$ )); small holotrichous a-isorhizas ( $n = 30$ ;  $4.26\text{--}5.46 \times 2.92\text{--}3.84 \mu\text{m}$  (mean =  $5.05 \times 3.43 \mu\text{m}$ )); holotrichous a-isorhizas ( $n = 30$ ;  $6.07\text{--}7.96 \times 4.59\text{--}5.92 \mu\text{m}$  (mean =  $5.51 \times 7.41 \mu\text{m}$ )); heterotrichous microbasic rhopaloids ( $n = 30$ ;  $12.40\text{--}14.48 \times 8.43\text{--}10.45 \mu\text{m}$  (mean =  $12.33 \times 9.51 \mu\text{m}$ )). Two other unidentified capsules were found (but see discussion below).



**Fig. 7.** Nematocyst types found in *Cassiopea mayeri*, sp. nov. and *Cassiopea culionensis*, stat. nov. (a) Undischarged heterotrichous microbasic rhopaloid (MZUSP 8633). (b) Discharged heterotrichous microbasic rhopaloid (MZUSP 8634). (c) Undischarged holotrichous a-isorhiza (MZUSP 8631). (d) Discharged holotrichous a-isorhiza (MZUSP 8633). (e) Undischarged small holotrichous a-isorhiza (MZUSP 8631). (f) Discharged small holotrichous a-isorhiza (MZUSP 8631). (g) Undischarged O-isorhiza (MZUSP 8631). (h, i) Unidentified cnidae capsules (MZUSP 8633). Scale bars: 5 µm.

### Female description (MZUSP 8633)

Formaldehyde preserved (Fig. 6d, 8a, b, d). Specimen in good condition, 11.30 cm in bell diameter. Exumbrella, mesoglea, marginal lappets, rhopalia number, subumbrellar musculature, oral disc, oral arms, gastrovascular cavity, gonads, radial canal system and colour of fixed specimen as in neotype.

**Oral appendages.** Oral disc with a central flattened parabolic appendage, 1.5 cm long, surrounded by numerous (sometimes folded), irregular, parabolic, oblong appendages of two sizes: smaller ones (0.2–0.4 cm long) that cover all arm disc; and bigger ones (1 cm long) in axil of some pairs of oral arms. Few small flat parabolic or orbicular appendages, ~1 mm long, over oral surface of mouth arms (especially in distal region). It is possible to observe eggs and planulae over the oral appendages (see Fig. 8c, e).

**Colour in life.** Ivory umbrella, dark greyish and ivory ventral surface of oral arms and ivory appendages with a cherry hue in some sections.

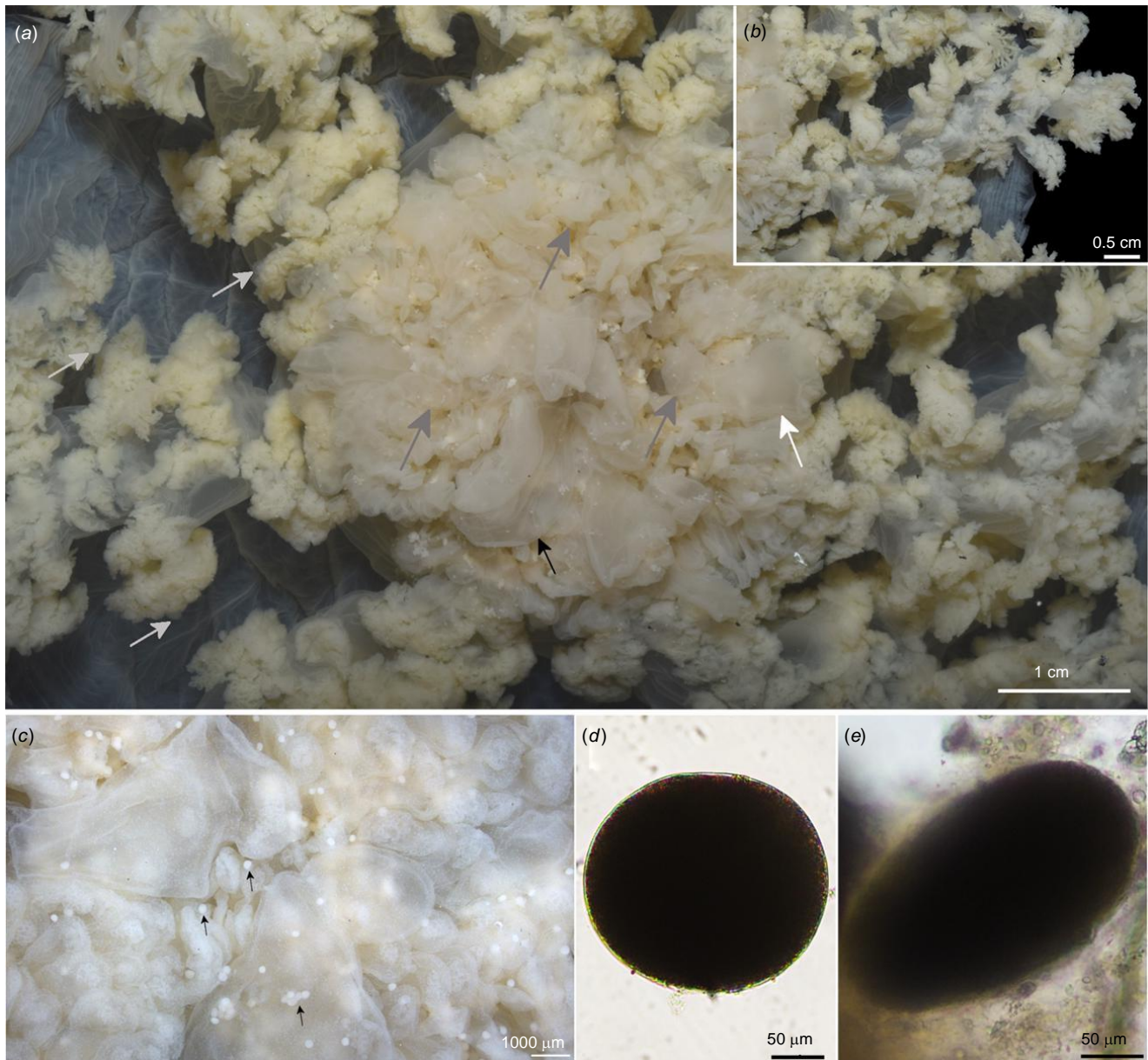
### Variation

Size 8.60–11.40 cm. Rhopalia not always equidistantly distributed; the closer they are, the lower the number of lappets between them, and vice versa; varying from 0 to ~6 per paramere; sometimes indistinct. Rhopalia number 11–20, 17 the most common value (three specimens of nine); ocellus present in some rhopalia (see Fig. 4d); rhopaliar pit covered by folding of two ocular lappets, which cover almost all except its base or that only cover its sides. Subgenital ostia

occasionally V-shaped. Oral arms usually eight, but sometimes seven. In some arms of the same individual, the distal bifurcation can start along the second quarter of the main trunk; in this case, there are ~3 lateral branches in alternate position along the central trunk of the arm. Males with linear appendages at the centre of the disc, instead of parabolic-shaped ones. In females, central appendages can be irregular, nearly rounded, parabolic, oblong- or oval-shaped; in some cases, they are folded, forming a ‘C’, and the apex can end in a distinct point. Gastrovascular cavity sometimes ovoid. Gonads sometimes forming a poorly defined cross-shape, and not filling fully the gastrovascular cavity. Canal system varies according to the number of sense organs, there are usually twice as many radial canals as rhopalia. The colouration of the central appendages can also be cream-greenish or greenish, and the cherry colouration can be in different extents along the central disc.

### Remarks

Based on specimens from the Philippines, Light (1914) described *C. polypoides* var. *culionensis*, a new variety of the Red Sea species *C. polypoides* Keller, 1883. Light (1914) stated that his variety was almost identical to *C. polypoides*, only differing in some features, such as the shape of the gastrovascular cavity and the presence of rhopalia without ocelli. However, Stiasny (1921) disregarded these features and considered *C. polypoides* and *C. polypoides* var. *culionensis* a synonym of *C. andromeda*. Later, Stiasny (1926) re-validated *C. polypoides* var. *culionensis*, but subsequent



**Fig. 8.** *Cassiopea culionensis*, stat. nov., formaldehyde-preserved females. (a) Oral disc (MZUSP 8633). Black arrow indicates the bigger central flattened parabolic appendage, dark grey arrows indicate the smaller appendages that cover all the arm disc and that surround the larger central one, light grey arrows indicate parabolic or orbicular appendages, white arrow indicates a parabolic appendage at the axil of a pair of oral arms. (b) Oral arm (MZUSP 8633). (c) Detail of the appendages at the central region of the oral disc (MZUSP 8640). Black arrows indicate eggs and planulae. (d) Egg (MZUSP 8633). (e) Planulae (MZUSP 8632).

studies re-synonymised it with *C. andromeda* (Kramp 1961; Jarms and Morandini 2019). Nevertheless, based on Light's description, the specimens we observed fit with the description of his variety, but also conform well with that of *C. andromeda*.

The description and subsequent revisions of *C. polypoides* var. *culionensis* raised two obvious hypotheses about its identity. The first scenario is that Light's 'variety' belongs to *C. andromeda*. This possibility is plausible if we consider the morphological similarity that exists among species of

*Cassiopea* (e.g. *C. andromeda* and *C. xamachana*, Hummelinck 1968) and the evidence of the multiple introductions of *C. andromeda* across the globe (Holland et al. 2004; Zenetos et al. 2011; Siokou et al. 2013; Morandini et al. 2017; Maggio et al. 2019; Stampar et al. 2020). This hypothesis is better supported if we consider that not all non-indigenous *Cassiopea* species were introduced recently (Morandini et al. 2017). A second scenario is that the samples might be incorrectly identified by Light and his jellyfish was not a variety of *C. polypoides* but rather a new species.

However, it is impossible to evaluate its relation to *C. polypoides* because, to our knowledge, the type material of the species was lost and unavailable for observations. We attempted to locate the type material in collections in the Philippines (University of the Philippines Biology Invertebrate Museum, and National Museum of the Philippines), where the material might conceivably have been found, and yet no specimens were located.

Further complicating matters, more than one species was probably included among the 27 specimens that Light used to describe his variety (see also Stiasny 1926). Light (1914) mentioned the existence of four morphotypes, including one with enlarged appendages on the arms. The presence of enlarged appendages could represent interspecific variability, a distinct possibility given that one of the taxa herein studied does not have enlarged appendages on the arms (*C. culionensis*, stat. nov.), but the other one does (*C. mayeri*, sp. nov.). Thus, not being able to link unambiguously Light's 'variety' (1914) to either the taxon herein discussed or to *C. andromeda*, we decided to use the epithet '*culionensis*' in redescribing and validating *C. polypoides* var. *culionensis* as *C. culionensis*, stat. nov. Light's (1914) description influenced our decision to validate *C. culionensis*, stat. nov. Most of his specimens were classified in three morphotypes that could represent the females and males classified here as *C. culionensis*, stat. nov. The morphotypes include one with a 'large central appendage and a whorl surrounding it' (probably our females), the second morphotype with 'a large central appendage without a surrounding whorl', and the third one without enlarged appendages (the last two could be our males) (Light 1914, p. 202). However, there are some caveats to our decision. For example, morphological delimitation can be inaccurate owing to the high level of overlap of characters between species (Gohar and Eisawy 1960; Morandini et al. 2017). In addition, our samples were collected in Lapu-Lapu, Cebu and *C. polypoides* var. *culionensis* was described from Culion Bay, Palawan, which might suggest a connection between these two localities.

Despite the absence of a holotype, or of paratype or even a syntype series from which to designate a lectotype (as well as the other caveats), we decided to take conservative action by resurrecting the name instead of portraying this taxon under a new name, designating the specimen MZUSP 8634 as the neotype specimen of the species *Cassiopea culionensis*, stat. nov. Although this specimen is not from the type locality (Culion Bay, Philippines), it came from a place (Lapu-Lapu, Philippines) close to the type locality (~450 km, straight-line distance). It is also in good condition and presents all the diagnostic characters described by Light (1914) for *C. polypoides* var. *culionensis*. *Cassiopea culionensis*, stat. nov. shares with *Cassiopea ndrosia* Agassiz & Mayer, 1899 and *C. ornata* the presence of small appendages; it differs from both by having larger appendages at the centre and at the base of each pair of oral arms; it also

differs from *C. ornata* in the shape of the appendages (not being club-shaped) and from *C. ndrosia* in the number of marginal lappets per paramere (five instead of four). *Cassiopea culionensis*, stat. nov. differs from *C. medusa* and *Cassiopea mertensi* (Brandt, 1838) in the number of marginal lappets per paramere (five instead of seven (*C. medusa*) or eight (*C. mertensi*)), and in the shape and general size of the oral appendages (most being small parabolic- or orbicular-shaped instead of very large ribbon-shaped (*C. medusa*) or very large clubs (*C. mertensi*)). Another point of difference from *C. medusa* is the number of distal branches (two instead of three).

### Biological data

Information about the life cycle of *C. culionensis*, stat. nov. is found in Mellas et al. (2014), since the authors mentioned that they used polyps and ephyrae in their assays. This species was recorded in sympatry with *C. mayeri*, sp. nov. in Lapu-Lapu, Cebu, Philippines and, according to Light (1914), with *C. medusa* in Culion Bay. As for other *Cassiopea* species, it harbours symbiotic dinoflagellates that have yet to be genotyped.

### Etymology

Named by Light (1914) after the type locality (Culion Bay, Philippines).

### Distribution

This species is known from Culion Bay and Lapu-Lapu, Philippines (Fig. 1).

## *Cassiopea mayeri*, sp. nov.

(Fig. 1, 7, 9–12)

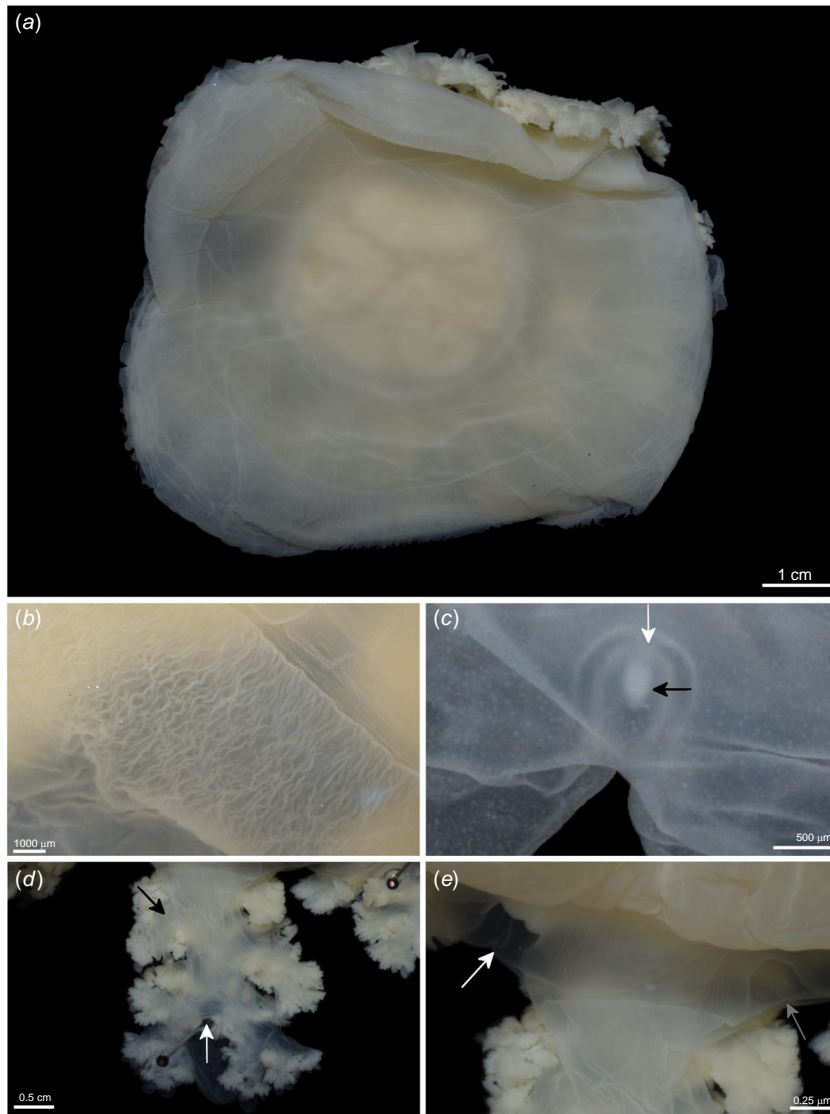
<http://www.zoobank.org/NomenclaturalActs/d2714142-7c01-4921-98c2-cc37c687dc7c>

*Type locality*: Lapu-Lapu, Cebu, Philippines (10.285967°N, 124.000750°E).

### Material examined

*Holotype* (MZUSP 8631). One male, 9.4 cm in bell diameter, from Lapu-Lapu, Cebu, Philippines, 10.285967°N 124.000750°E; coll. S. M. Geson III, hand captured, 01.iii.2019, 4% formaldehyde solution in seawater; DNA subsample – ethanol preserved – voucher ID gACM 00339.

*Paratypes*. UF 009664 (CASIZ 201000): identified as *Cassiopea* sp., one young female medusa, 3.5 cm in diameter, from Bumbon Reef, Calatagan, Luzon Island, Philippines, 13.90801°N 120.60553°E; coll. G. Paulay and D. Uyeno, 09.v.2014, Menthol → 10% formaldehyde → 75% ethyl alcohol; DNA subsample – ethyl alcohol preserved – voucher ID UF 009664. UF 009665 (CASIZ 200996): identified as *Cassiopea* sp., one young medusa, 3.2 cm in diameter, from S of Caritunan Reef,



**Fig. 9.** *Cassiopea mayeri*, sp. nov., aboral views of the formalin preserved male holotype (MZUSP 8631). (a) Exumbrellar (aboral) view. (b) Exumbrellar raised ring (*ringwulst*). (c) Exumbrellar view of a rhopalium and rhopalial pit. Black arrow indicates a rhopalium, white arrow indicates the rhopalial pit (wide bowl shape). (d) Branching pattern of the oral arm. Black arrow indicates a lateral branch, white arrow indicates the beginning of the terminal bifurcation. (e) Subgenital ostium and perradial notch. Grey arrow indicates a perradial notch, white arrow indicates a subgenital ostium.

Calatagan, Luzon Island, Philippines, 13.90431°N 120.60535°E; coll. VIP team, 10.v.2014, 10% formaldehyde → 75% ethyl alcohol; DNA subsample – ethyl alcohol preserved – voucher ID UF 009665. UF 007505: cnidaria identified as *Cassiopea* sp., one young medusa, 2.13 cm in diameter, from Uehara Harbor, Iriomote Island, Ryukyu Islands, Okinawa Prefecture, Japan, 24.41895°N 123.80476°E; coll. N. Evans, F. Michonneau and T. Naruse, 11.vii.2010, 10% formaldehyde → 75% ethyl alcohol; DNA subsample – ethyl alcohol preserved – voucher ID UF 007505.

*Other material examined.* USNM 27941: identified as *Cassiopea ornata* (variety of), seven medusae, 3.6–7.85 cm in diameter, from Olongapo, Luzon Island, Philippines; coll. USA Bureau of Fisheries, Albatross Philippine Expedition (1907–1908), 07.i.1908, 4% formaldehyde.

### Diagnosis (but see also Discussion)

Oral arms with small parabolic or orbicular appendages throughout, and large ones at centre of disc, at axil of each pair of arms and at distal ends of arms. Tuber-shaped

appendages on oral disc of larger specimens, and irregularly fungiform appendages in the central disc of preserved female specimens; round rhopalial pit; five marginal lappets per paramere.

### Holotype description (MZUSP 8631)

Specimen in good condition, 9.40 cm in bell diameter.

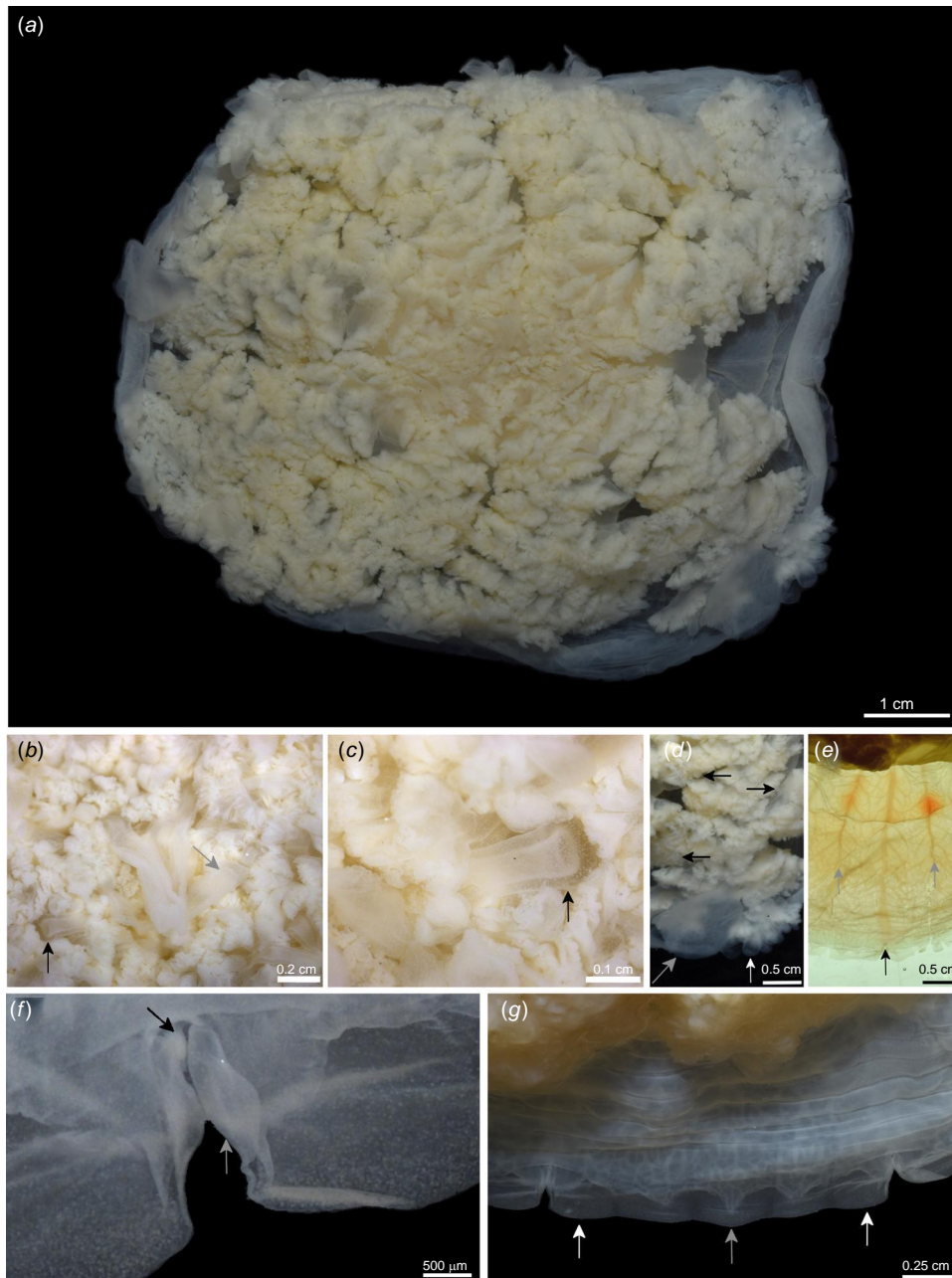
*Exumbrella* (Fig. 9a, b). Translucent and slightly bulging in central region, slightly depressed around bulging region, gradually rising to exumbrellar raised ring (*ringwulst*); smooth, except for *ringwulst*.

*Mesoglea*. Flexible, thinner at edge.

*Marginal lappets* (Fig. 10g). Five per paramere (three velar flanked by two ocular), blunt with central one larger.

*Rhopalia* (Fig. 9c, 10f). Sixteen; from exumbrellar side, each sense organ in a rounded, wide bowl-like rhopalial pit; ocelli not observed (maybe due to preservation); from





**Fig. 10.** *Cassiopea mayeri*, sp. nov., oral views of the formaldehyde-preserved male holotype (MZUSP 8631). (a) Subumbrellar (oral) view. (b) Detail of the appendages at the oral disc. Black arrow indicates a tuber-shaped appendage, grey arrow indicates a linear-shaped appendage in the central region. (c) Tuber-shaped appendage. (d) Appendages of the oral arm. Black arrows indicate small orbicular or parabolic appendages, grey arrow indicates the bigger parabolic nearly rounded appendage at the axil of a terminal bifurcation, white arrow indicates a more elongated (oblong to parabolic in shape) appendage at the distal end of an arm. (e) Canal system showing some of the rhopalial and inter-rhopalial canals and anastomoses. Black arrow indicates a rhopalial canal, grey arrows indicate inter-rhopalial canals. (f) Rhopalium and ocular lappets. Black arrow indicates a rhopalium, grey arrow indicates the folding of an ocular lappet covering the rhopalial pit. (g) Subumbrellar view of the bell margin showing a paramere. Grey arrow indicates a velar lappet, white arrows indicate ocular lappets.

subumbrellar side, rhopalial pits are partially covered by folding of two ocular lappets.

**Subumbrellar musculature.** Repeated pattern of fine U- or V-shaped muscle bands.

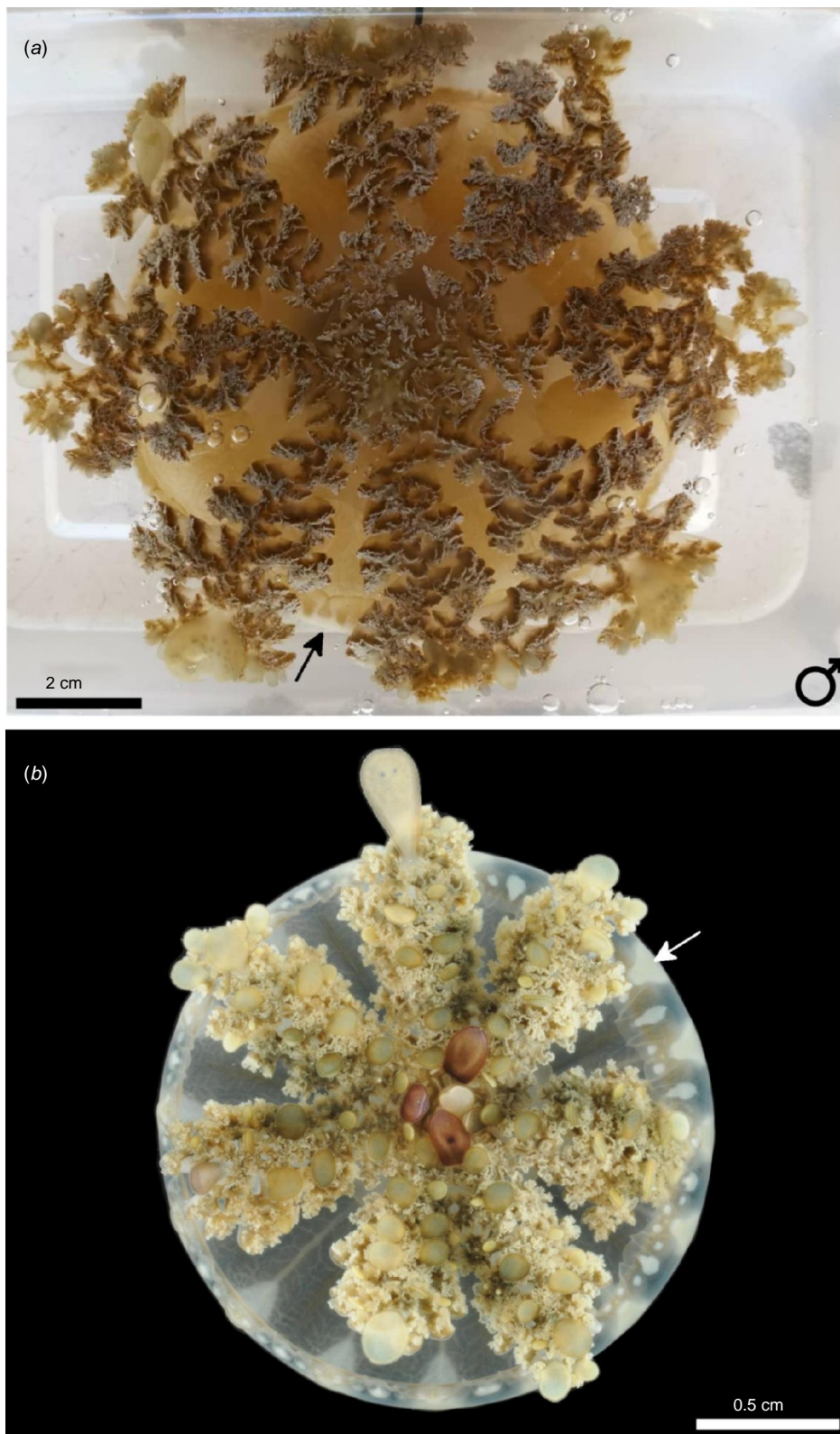
**Subgenital ostia** (Fig. 9e). Four small, interradian, U-shaped, 0.42 cm wide and 0.30 cm high; with three shallow perradial notches, U- or V-shaped.

**Oral arm disc** (Fig. 10a). Flat, almost as wide as bell radius, 4.26 cm wide.

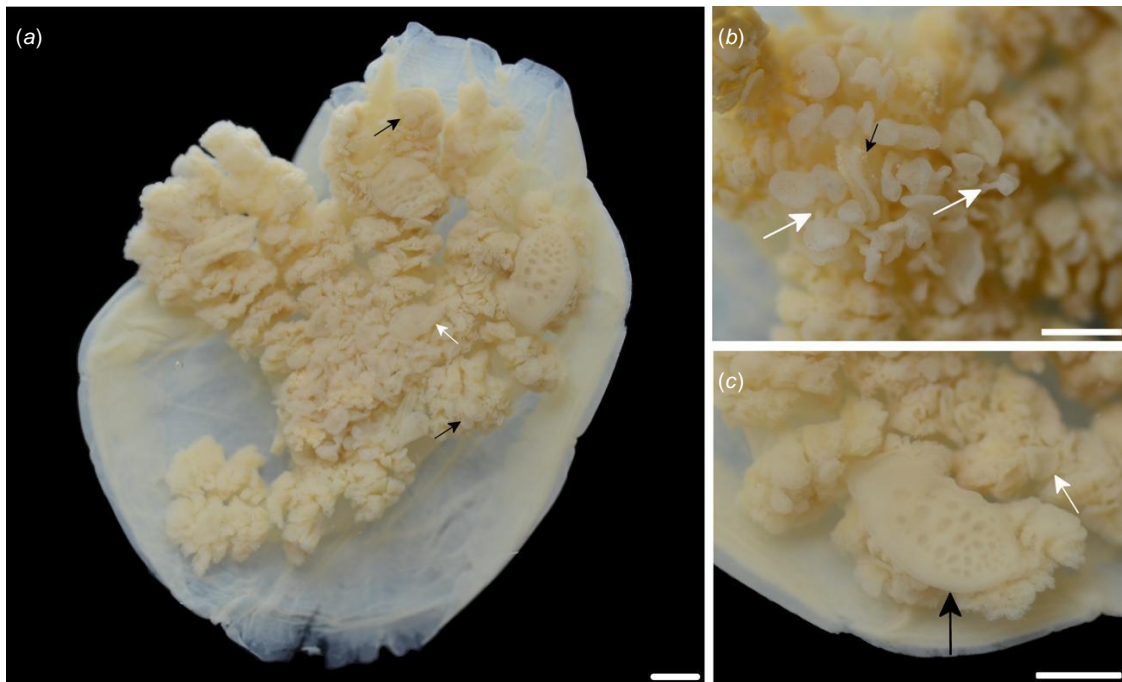
**Oral arms** (Fig. 9d, 10a). Eight mouth arms (four pairs), in adradial position, arise from oral disc at centre of subumbrella. Oral arms variable in size, but usually

approximately one-third bell diameter, 2.93 cm in length, can extend a little beyond bell margin. Approximately 3–5 lateral branches alternate in position along central trunk of arm; central trunk ends in a bifurcation that starts before last quarter of main trunk.

**Oral appendages** (Fig. 10a–d). Oral disc with one central linear appendage, 8 mm long, and 1–2 similar appendages in axil of each pair of arms; oral disc also has one tuber-shaped appendage, 3 mm long. Small orbicular- and parabolic-shaped appendages along oral surface of arms, 1–1.5 mm long; distal end of arms with several appendages that tend to be more elongated (oblong and parabolic in



**Fig. 11.** *Cassiopea mayeri*, sp. nov., oral view of live specimens. (a) Male holotype (MZUSP 8631). (b) Young medusa paratype (UF 007505), photo from Florida Museum of Natural History – Invertebrate Zoology. Arrows indicate whitish marks in the exumbrella.



**Fig. 12.** *Cassiopea mayeri*, sp. nov., oral views of the formaldehyde-preserved female paratype (UF 009664/CASIZ 201000). (a) Oral view of the young female medusa. Black arrows indicate orbicular appendages, white arrow indicates a spatulated appendage. (b) Detail of the appendages at the central region of the oral disc. Black arrow indicates a group of eggs and planulae, white arrows indicate fungiform (peltate) appendages. (c) Appendages found in oral arms. Black arrow indicates the bigger, oblong appendage at the axil of the terminal bifurcation, white arrow indicates an orbicular appendage. Scale bars: 0.25 cm.

shape), 2–5 mm long; terminal bifurcation of some arms with a big parabolic (nearly rounded)-shaped appendage, 9 mm long.

**Gastrovascular cavity** (Fig. 9a). Circular, 3.59 cm in diameter.

**Gonads** (Fig. 9a). Four gonads are spread out forming a not well-defined cross-shaped shape within stomach.

**Canal system** (Fig. 10e). A main canal extends from each of eight oral arms into gastrovascular cavity, where 32 radial canals arise and go along subumbrella, communicating by a network of anastomosing vessels; 16 radial canals end in a rhopalium (rhopalial canals) and 16 are intermediate in position (inter-rhopalial canals).

**Colour in 4% formaldehyde** (Fig. 9a, 10a). Translucent whitish umbrella, oral arms, central disc and appendages; creamish-yellow gonads and ventral surface of oral arms.

**Colour in life** (Fig. 11a). Yellowish-amber umbrella, dark amber-greyish ventral surface of oral arms (oral groove), and whitish and whitish-amber appendages. Exumbrellar colour not documented; however, whitish marks were observed in photographs taken of the live specimen (Fig. 11a), which faded after fixation.

**Nematocysts** (for nematocyst identifications see Fig. 7). Oral appendages with holotrichous O-isorhizas ( $n = 3$ ;  $7.47\text{--}7.90 \times 6.27\text{--}6.63 \mu\text{m}$  (mean =  $6.39 \times 7.72 \mu\text{m}$ ));

small holotrichous a-isorhizas ( $n = 30$ ;  $4.26\text{--}5.33 \times 2.37\text{--}3.29 \mu\text{m}$  (mean =  $4.80 \times 2.82 \mu\text{m}$ )); holotrichous a-isorhizas ( $n = 30$ ;  $6.79\text{--}8.06 \times 5.25\text{--}6.15 \mu\text{m}$  (mean =  $5.77 \times 7.36 \mu\text{m}$ )); heterotrichous microbasic rhopaloids ( $n = 30$ ;  $12.20\text{--}13.62 \times 9.25\text{--}11.21 \mu\text{m}$  (mean =  $12.92 \times 9.98 \mu\text{m}$ )). Two other unidentified capsules were found, as in *C. culionensis*, stat. nov.

### Female description (UF 009664/CASIZ 201000)

Preserved in 75% ethyl alcohol (Fig. 12a–c). Specimen with six oral arms (two of them excised), folded umbrella in regular condition of preservation, 3.25 cm in bell diameter. Exumbrella, mesoglea, marginal lappets, subumbrellar musculature, ocelli, oral arms, gastrovascular cavity and colour as in the holotype.

**Rhopalia.** Ten, not documented with precision owing to preservation condition of bell; no trace of ocelli found.

**Oral disc.** Flat, almost one-third of bell radius, 0.94 cm wide.

**Oral appendages.** Oral disc densely covered with spatulated and fungiform (peltate) appendages (0.5–1.5 mm long), with bigger ones at centre of disc and axil of some pairs of arms (up to 3.5 mm long). Small flat orbicular appendages (0.5–1.5 mm long) over oral surface of mouth arms; axil of terminal bifurcation of some arms with a bigger

appendage, oblong-shaped, 5 mm long. It is possible to observe eggs and planulae over the oral appendages. Gonads not visible. Canal system not documented.

*Colour in life.* Not documented.

## Variation

Size: 2.13–9.40 cm. The velar lappets contiguous to the ocular ones can be pointed. Rhopalia not always equidistantly distributed; the closer they are, the lower the number of lappets between them, and vice versa; varying from 0 to 6 velar lappets per paramere. Subgenital ostia sometimes V-shaped. Oral arms usually eight, but sometimes seven. Oral appendages in smaller specimens not visible, at least to the naked eye, tuber-shaped appendages. Compared with the holotype, the parabolic or orbicular appendages along the oral arms in the specimen from Japan are more conspicuous and not ‘translucent’ but opaque; the oral appendages appear in three different colours: cherry, yellowish-green and creamish-yellow; and the oral groove appears in two colour zones, grey (at the inner side of each pair of arms) and creamish-yellow (in the outer side of each pair of arms). The bigger appendage of the terminal bifurcation can be orbicular, oblong- or tear-shaped.

## Remarks

In 1908, A. G. Mayer identified seven specimens (USNM 27941) from Olongapo, Luzon Island, Philippines (~100 km from the collection place of UF 009664/CASIZ 201000 and UF 009665/CASIZ 200996), labelled as ‘*C. ornata* (variety of)’. The larger of the specimens (Supplementary Fig. S4; female, 7.85 cm in diameter) resembles *C. mayeri*, sp. nov. because, as in the holotype, it has tuber-shaped appendages (a character never before documented in any species of *Cassiopea*). Furthermore, as in UF 009664/CASIZ 201000, it has fungiform (peltate) appendages in the oral disc and the remains of small appendages along the oral arms. Moreover, from the exumbrellar side, the rhopalia are in rounded, wide bowl-shaped rhopalial pits. However, it does not have appendages at the distal bifurcation of some arms (maybe due to preservation) as observed in our type material. Owing to the lack of all the diagnostic characters of *C. mayeri*, sp. nov., we identified this specimen as *Cassiopea* aff. *mayeri* (Supplementary Fig. S4).

*Cassiopea mayeri*, sp. nov. shares with *C. culionensis*, stat. nov. the small, parabolic or orbicular appendages over the ventral surface of the arms; it differs from *C. culionensis*, stat. nov. by the shape of the rhopalial pit and it has big appendages at the tip of the oral arms while *C. culionensis*, stat. nov. does not; in addition, *C. culionensis*, stat. nov. has slender and larger oral arms. *Cassiopea mayeri*, sp. nov. shares with *C. ndrosia* and *C. ornata* the presence of small appendages; it differs from both of them by having large appendages at the centre, at the base of each pair of oral

arms, and at the axis of the terminal bifurcation of the oral arms; it also differs from *C. ornata* in the shape of the small appendages (orbicular instead of club-shaped), and from *C. ndrosia* in the number of marginal lappets per paramere (five instead of four). *Cassiopea mayeri*, sp. nov. differs from *C. medusa* and *C. mertensi* in the number of marginal lappets per paramere (five instead of seven (*C. medusa*) or eight (*C. mertensi*)), and in the shape and general size of the oral appendages (most being small parabolic- or orbicular-shaped instead of very large ribbon-shaped (*C. medusa*) or very large clubs (*C. mertensi*)). *C. mayeri*, sp. nov. also differs from *C. medusa* in the number of distal branches (two instead of three).

Based on our molecular results, previous studies have included molecular data (*COI*) of this species under different names:

- *Cassiopea andromeda* – Ojimi and Hidaka (2010), p. 2280 (GenBank accession numbers: AB563739–40; telomere length among different life cycle stages of *Cassiopea*); Galil et al. (2010), p. 333 (AB563740 used in a phylogenetic analysis); Miller et al. (2012), p. 428 (AB563740 used in a phylogenetic analysis); Prieto et al. (2013), p. 3245 (AB563740 used in a BLAST analysis for the estimation of mean identity values between *Rhizostoma luteum* and other jellyfish belonging to the order Rhizostomeae).
- *Cephea* sp. 2 – Galil et al. (2017), p. 231 (AB563740 used in a phylogenetic analysis).
- *Cassiopea* sp. 5 – Arai et al. (2017), p. 136 (AB563739–40; list of updated names of species of *Cassiopea*); Maggio et al. (2019), p. 2 (AB563739–40 used in a phylogenetic analysis).

## Biological data

This taxon was used by Ojimi and Hidaka (2010) to improve understanding of the mechanisms underlying life spans of cnidarian polyp and medusa stages, showing that chromosomes within cells of the umbrella of the jellyfish are defined by longer telomeres than those of polyps, free swimming buds or other regions of the medusa. Data about its life cycle are found in the same study, since the authors used free swimming buds, scyphistomae and medusae in their assays. This species was recorded in sympatry with *C. culionensis*, stat. nov. in Lapu-Lapu, Cebu, Central Visayas, Philippines. As for other *Cassiopea* species, it harbours symbiotic dinoflagellates that have yet to be genotyped.

## Etymology

Named after Alfred G. Mayer for his invaluable contributions to jellyfish taxonomy. He examined specimens of *Cassiopea* from all over the world and documented tuber-like appendages in medusae identified as ‘a closely related variety of, if not identical with, *C. ornata*’ from the

Philippines (Mayer 1910, p. 648), which is most likely *C. mayeri*, sp. nov.

## Distribution

This species is known from Calatagan and Lapu-Lapu, Philippines, and Iriomote Island, Japan (Fig. 1).

## Discussion

### Phenotypic diagnosis

Here, we described *C. mayeri*, sp. nov. and redescribed and validated *C. culionensis*, stat. nov., increasing the number of valid species to 12 (Jarms and Morandini 2019). Even when other authors have found that phenotypic characters can be of limited taxonomic utility (Maas 1903; Gohar and Eisawy 1960; Hummelinck 1968), we were able to differentiate one species from the other using morphological features. In scyphomedusae, taxonomic studies with large sample sizes tend to detect morphological variation that compromise the reliability of characters previously considered to be diagnostic (Keller 1883; Gohar and Eisawy 1960; Kramp 1968; Dawson 2005b; Lawley et al. 2021). Thus, the inspection of a larger number of individuals is needed to elucidate the intraspecific phenotypic variation of both species, and to test the unambiguity of the proposed diagnostic characters. We also need to increase the sampling effort of jellyfishes in the Western Pacific, since other synonymised taxa (e.g. '*Cassiopeja*' *acycloblia* Schultze, 1898 synonym of *C. andromeda*) from the region could support or invalidate the morphological distinction among related species in close geographic proximity.

Among the upside-down jellyfishes (*Cassiopea*), previous efforts used the number of rhopalia, unsuccessfully, to create taxonomic classifications at supra-generic level (Haeckel 1880) and to delimit species (Gohar and Eisawy 1960). In this study, we included information, for the first time, about the rhopaliar pit shape from an exumbrellar view in *Cassiopea*, which was useful to distinguish two sympatric species in Lapu-Lapu, Cebu, Philippines. The use of characters related to the sense organs, in a traditional context, have allowed species of *Aurelia* from the Mediterranean to be distinguished based on differences in the shape of the dorsal hood that protects the sense organ and by the direction of the rhopalium (Scorrano et al. 2016). It is important to emphasise that although discrete characters are useful for establishing boundaries between taxa, the delimitation of species can be improved by integrating multivariate analyses of continuous and meristic morphological characters (Dawson 2003; Piraino et al. 2014; Chiaverano et al. 2016; Gómez Daglio and Dawson 2017; de Souza and Dawson 2018). For example, analysed under a statistical framework, the variables rhopaliar lappets shape, velar lappets shape, rhopalia position, presence of bifurcated velar lappets and

number of bifurcated velar lappets are among the characters that most contribute to distinguishing species of *Chrysaora* Péron & Lesueur, 1810, *Lychnorhiza* Haeckel, 1880 and *Sanderia* Goette, 1886 (Gómez Daglio and Dawson 2017).

Historically, as in other related genera (e.g. *Mastigias* Agassiz, 1862 and *Thysanostoma* Agassiz, 1862) (Kramp 1961), the size, number and shape of oral appendages have been used to identify species of *Cassiopea* (Mayer 1910; Kramp 1961; Gershwin et al. 2010; Keable and Ah Yong 2016). Regarding our results, morphological observations of the appendages allowed us to support the hypothesis that our specimens could be classified as two different species. Unfortunately, historical species descriptions do not all provide illustrations of appendages. There is no information about the variation in the appendages between males and females. Finally, there is no standardised terminology to refer to the shapes of appendages found in *Cassiopea* or even Rhizostomeae in general. These deficiencies hamper comparison among the appendages of all the species of the genus and therefore compromise the utility of features related to the oral appendages as diagnostic characters. For example, the presence of tuber-shaped appendages in *C. mayeri*, sp. nov. have never before been recorded. To lessen the chaotic systematics of *Cassiopea*, we used a terminology based on an illustrated dictionary of botanical terms (Lindley 1832) to refer to appendage shapes observed in *C. mayeri*, sp. nov. and *C. culionensis*, stat. nov. It was not our intention to review or standardise the nomenclature of the appendages of all the species of *Cassiopea* at this time, but to draw attention to the lack of standardised terminology (de Souza and Dawson 2018).

The cnidome has been included in species descriptions of some Semaestomeae jellyfish in recent years (Matsumoto et al. 2003; Raskoff and Matsumoto 2004; Gershwin and Zeidler 2008a; Mutlu et al. 2020; Ras et al. 2020); however, none of the original species descriptions of Rhizostomeae from, at least, the last 80 years has nematocyst data (Moestafa and McConnaughey 1966; Pagès et al. 1992; Gershwin and Zeidler 2008b; Galil et al. 2010; Gershwin et al. 2010; Gershwin and Davie 2013; Nishikawa et al. 2015). In the case of *Cassiopea*, there are data on the cnidome of *C. andromeda* and *C. xamachana*: oval a-isorhizas, round O-isorhizas and oval-shaped birhopaloids (Jensch and Hofmann 1997; Östman 2000; Heins et al. 2015; Gülşahin 2016; Ames et al. 2020). Conversely, small lemon-shaped birhopaloids have been found only in *C. andromeda* (Heins et al. 2015). In *C. mayeri*, sp. nov. and *C. culionensis*, stat. nov., we recognised oval a-isorhizas (two size classes), round O-isorhizas (rare) and oval-shaped birhopaloids. Additionally, we observed two unidentified undischarged capsules (Fig. 7h, i), which resemble large immature A-isorhiza (see Östman and Hydman 1997, Fig. 7d) or large A-isorhiza (see Avian et al. 2016, Fig. 10e). A more detailed study on the cnidome of the medusae stage of *C. mayeri*, sp. nov., *C. culionensis*, stat.

nov. and *C. xamachana* could help to confirm the presence or absence of small lemon-shaped rhopaloids. In the same way, a review of the nematocyst content of freshly collected *C. andromeda* and *C. xamachana* medusae could help to confirm the presence or absence of the unidentified undischarged capsules found in *C. mayeri*, sp. nov. and *C. culionensis*, stat. nov. If those absences were confirmed, they would be useful taxonomical characters to establish boundaries among *Cassiopea* species.

This study provides further evidence of sympatry within the genus *Cassiopea*, because *C. mayeri*, sp. nov. and *C. culionensis*, stat. nov. occur together in the Lapu-Lapu region. Similarly, *C. culionensis*, stat. nov. and *C. medusa* occur in Culion Bay (Light 1914), *C. andromeda* and *C. frondosa* co-occur in Key Largo, Florida Keys (Holland et al. 2004), and *Cassiopea* sp. 4 and *Cassiopea* sp. 6 in 'NGE Lake 1 Lagoon', Palau (Arai et al. 2017). Likewise, *C. andromeda* and *Cassiopea depressa* Haeckel, 1880 were registered in close proximity in Madagascar and Mozambique (Haeckel 1880; Stiasny 1931). Finally, *C. frondosa* and *C. xamachana* are reported in several localities of the Americas (Hummelink 1968; Larson 1982; Collado-Vides et al. 1988; Larson 1997; D'Ambra et al. 2015; Gómez Daglio and Dawson 2017). The widespread occurrence of multiple congeneric species in an area, like the case of *Cassiopea*, calls for the development of regional taxonomic keys and records of habitat preference (Glasby et al. 2019). Habitat preference information combined with other types of evidence can improve species identification of *Cassiopea*, although only a few records show habitat preferences of *Cassiopea* medusae (i.e. for *C. frondosa* and *C. xamachana* in Florida Keys and Jamaica) (Mayer 1910; Larson 1997). Thus, given the importance of habitat information for jellyfish, we must record ecological data about collection sites for *Cassiopea* in future surveys.

Revising the taxonomy of *Cassiopea* is essential to corroborate the putative cryptic status of the genus and, as aforementioned, to define the range of morphological characters to differentially diagnose species within *Cassiopea*. If diagnostic characters for at least some species exist, it would be possible to identify old specimens from museum collections preserved in formaldehyde and ethyl alcohol; some of which might be difficult to collect again. Additionally, recent observations of specimens collected in Palau suggest that body colouration is similar among different species inhabiting marine lakes and that various body colours were observed among *Cassiopea* species inhabiting a lagoon (Arai et al. 2017). Therefore, studying the morphology of *Cassiopea* species could help us understand how phenotypic variation can be environmentally induced (Chiaverano et al. 2016; Swift et al. 2016) and the evolutionary processes that led to low levels of morphological disparity (Struck et al. 2018). However, achieving that level of knowledge will require the integration of other sources of evidence, notably molecular data.

## Molecular evidence and integrative approaches

Despite certain advances achieved with morphological approaches, it is clear even for systematists of different generations and levels of training, that morphology alone is not enough to solve all the evolutionary species issues. In this sense, the use of molecular approaches (integrated or not with other types of evidence) has provided improvements in the study of the Scyphozoa by helping to detect cryptic diversity and refining the systematics of various non- and morphologically diagnosable lineages (e.g. Dawson 2005c, Holst and Laakmann 2014; Gómez Daglio and Dawson 2017). In the case of *Cassiopea*, analyses of *COI* sequences helped to detect cryptic taxa and invasive species (Holland et al. 2004; Gómez Daglio and Dawson 2017; Morandini et al. 2017; Maggio et al. 2019). Moreover, *COI* information (including ours) supports 5 candidate species and 6 of the 12 accepted species (*Cassiopea* sp. 1–4, *Cassiopea* sp. 6, *C. andromeda*, *C. culionensis*, stat. nov., *C. frondosa*, *C. mayeri*, sp. nov., *C. ornata* and *C. xamachana*) (Holland et al. 2004; Arai et al. 2017; Gamero-Mora et al. 2019).

The intraspecific genetic divergences we computed from *16S* and *COI* sequences for *C. mayeri*, sp. nov. and *C. culionensis*, stat. nov. were lower than the interspecific divergences. The genetic distances we calculated for *COI* are within the range of conspecifics and congeners for other rhizostome jellyfishes. For example, for *Lychnorhiza* species from the Americas, the mean K2P intraspecific pairwise distance is  $0.005 \pm 0.004$ , and the average interspecific distance  $0.129 \pm 0.01$  (Gómez Daglio and Dawson 2017). Values among Asian rhizostome clades are similar or smaller; for example, for *Mastigias* the average within-clade distance is  $0.010 \pm 0.004$  for the China Sea (CHS) group,  $0.010 \pm 0$  for Solomon Sea (SS), and  $0.007 \pm 0.003$  for the Tropical West Pacific clade (TWP); and the average between-clade distance is  $0.086 \pm 0.032$  for CHS-SS,  $0.085 \pm 0.022$  for TWP-SS and  $0.071 \pm 0.031$  for the TWP-CHS clade (de Souza and Dawson 2018). Regarding pairwise genetic distances computed using *16S* sequences, *C. mayeri*, sp. nov. and *C. culionensis*, stat. nov. are separated by genetic divergences equivalent to species and genus-level differences in Semaestomeae jellyfishes (Avian et al. 2016; Rizman-Idid et al. 2016; Bayha et al. 2017). The intra- and interspecific genetic measures, including the results presented herein, need to be interpreted cautiously, since they should not be used to delimit species (Lawley et al. 2021).

The phylogenetic reconstructions based on *16S* and *COI* fragments provide consistent evidence that *C. mayeri*, sp. nov. and *C. culionensis*, stat. nov. are distinct species. The phylogeny of *Cassiopea* inferred using *16S*, which was used for the first time, gives a well-resolved picture of the relationships within this genus, but there are low bootstrap values on the three innermost nodes (<70%, Supplementary Fig. S1b). Indeed, low bootstrap values on internal nodes have been found in previously published *COI*

phylogenetic reconstructions of this genus (Holland *et al.* 2004; Arai *et al.* 2017; Morandini *et al.* 2017; Gamero-Mora *et al.* 2019; Maggio *et al.* 2019). Incongruous with ours (Supplementary Fig. S2b) and previous *COI* trees (Holland *et al.* 2004; Morandini *et al.* 2017; Gamero-Mora *et al.* 2019), and in agreement with our combined analyses (Fig. 2, Supplementary Fig. S3) and other phylogenies (Arai *et al.* 2017; Maggio *et al.* 2019), our 16S reconstruction shows the formation of a clade with only upside-down jellyfishes from the Indo-Pacific region (Supplementary Fig. S1). However, a *COI* tree that we constructed using exactly the same taxon sampling as 16S (i.e. excluding *Cassiopea* sp. 1–4 and *Cassiopea* sp. 6 from the *COI* dataset) also supports the presence of a clade with only *Cassiopea* species from the Indo-Pacific region (data not shown). Consequently, the topology we obtained with reduced 16S taxon sampling could change with the inclusion of *Cassiopea* sp. 1–4 and *Cassiopea* sp. 6 in the analysis; therefore, its addition is essential to confirm our results.

Even when some relationships between clades differ in individual and combined analyses, the species hypotheses are the same. In our combined analysis, there are low clade stability values on some internal nodes (Fig. 2). Therefore, the addition of 16S sequences for *Cassiopea* sp. 1–4 and *Cassiopea* sp. 6 in the combined analysis could result in alternative topologies with higher clade stability values. Indeed, we can observe higher clade stability values in internal nodes when singletons and terminals with missing data are excluded from the combined analysis (Supplementary Fig. S3). As prevalence of incomplete taxon sampling is a problem common in DNA-based species delimitation studies (Lim *et al.* 2012; Mastrototaro *et al.* 2020), we encourage the generation of data from different markers, including 16S. Moreover, nuclear markers could help to provide more robust inferences in the internal branching within *Cassiopea*. Nuclear markers could also be useful to delineate *C. mayeri*, sp. nov. and *C. culionensis*, stat. nov. better, as was done for some other scyphomedusae (Bayha and Dawson 2010; Avian *et al.* 2016; Bayha *et al.* 2017).

Besides the advances in phylogeny and species discovery, molecular data have led to the study of some other aspects of scyphozoan biology; for example, discoveries in population genetics (Dawson 2005c; Stopar *et al.* 2010; Ramšak *et al.* 2012; Ale *et al.* 2019). Our results based on 16S and *COI* sequences revealed the presence of three and seven haplotypes within *C. culionensis*, stat. nov. respectively. Regarding *C. mayeri*, sp. nov., five haplotypes were revealed for both 16S and *COI* (which are divided in two major haplotype groups, one from the Philippines and another from Japan); with a 16S haplotype shared among specimens from western Luzon and Central Visayas, which suggest that the populations of both localities are connected genetically. Previous studies on the population genetics of scyphozoans, using a variety of genetic markers and different geographic scales, have shown the existence (Dawson 2005c; Getino

Mamet *et al.* 2019) or lack of population genetic structure among populations (Stopar *et al.* 2010; Ramšak *et al.* 2012). The existence and the lack of population structure in those jellyfishes have been associated with life cycle, habitat specificities or the presence or absence of geographic barriers. In the case of *Cassiopea* species, the polyp stage is sessile, the planuloid bud, planula and ephyra stages are pelagic, and the jellyfish stage is an epibenthic gonochoristic brooder (Ohdera *et al.* 2018). As *Cassiopea* has not been studied under a population genetic approach, it is not known if some parts of its life cycle, or other factors, affect their genetic structure. However, with the increased interest in the study of *Cassiopea* species (Medina *et al.* 2021), the generation of new data, the emergence of new sequencing technologies and new analytical approaches, it will be possible to get robust results for the population genetics and phylogeography of species of the genus.

Incorporating molecular information for *Cassiopea* in studies at larger evolutionary scales allowed a better understanding on the origin and evolution of envenomation strategies (Ames *et al.* 2020) and on the origins of key organismal traits (Kayal *et al.* 2018). However, a proper integration of molecular and morphological data for studying *Cassiopea* systematics does not exist. Using these approaches separately produced, besides the big achievements accomplished, an inaccurate morphological delimitation with the description of species for which taxonomic validity has not been evaluated (Kramp 1961; Jarms and Morandini 2019), and the creation of candidate species (i.e. numbered *Cassiopea*) based on molecular data (Holland *et al.* 2004; Arai *et al.* 2017). To overcome the shortages of each type of evidence, we need to propose species hypotheses using different types of characters by embracing an integrative taxonomical approach, like the one used in other cryptic groups (Jörger and Schrödl 2013; Trevisan *et al.* 2017). Here, an integrative framework – specifically the congruence of molecular and phenotypic evidence – showed that the species richness of *Cassiopea* species in the Western Pacific is underestimated. Therefore, this study can serve as reference for future examinations of *Cassiopea* and other allied taxa. More research is necessary to solve the challenging systematics of the upside-down jellyfishes – including testing of all the species hypotheses by considering new sources of evidence. Nevertheless, our present study takes us one step further.

## Supplementary material

Supplementary material is available [online](#).

## References

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**Data availability.** The data underlying this study have been uploaded to GenBank (MW160911–MW160937, MW164859–MW164886) and FigShare (<https://doi.org/10.6084/m9.figshare.16528203>).

**Conflicts of interest.** The authors declare that they have no conflicts of interest.

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## Supplementary Material

### **Revealing hidden diversity among upside-down jellyfishes (Cnidaria: Scyphozoa: Rhizostomeae: *Cassiopea*): distinct evidence allows the change of status of a neglected variety and the description of a new species**

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**Table S1. Mitochondrial ribosomal gene *16S* rRNA and mitochondrial protein-encoding gene cytochrome *c* oxidase I (*COI*) sequences used for combined-marker analysis**

Terminal ID corresponds to terminal names on maximum likelihood tree based on *16S+COI* (Fig. 2).

–, marker not available for the taxa. GenBank accession numbers of sequences obtained in this study are in bold

Terminal ID	<i>16S</i>	<i>COI</i>
<i>Cassiopea andromeda</i> – French Polynesia	JN700934	JN700934
<i>C. andromeda</i> – Mexico	KY610609	KY610551
<i>C. culionensis_1</i> – Cebu, Philippines	<b>MW164879</b>	<b>MW160923</b>
<i>C. culionensis_2</i> – Cebu, Philippines	<b>MW164869</b>	<b>MW160913</b>
<i>C. culionensis_3</i> – Cebu, Philippines	<b>MW164886</b>	<b>MW160930</b>
<i>C. frondosa</i> – Florida, USA	KY610617	AY319467
<i>C. frondosa</i> – Panama	–	KY610560
<i>C. mayeri</i> – Japan	<b>MW164859</b>	<b>MW160931</b>
<i>C. mayeri_1</i> – Cebu, Philippines	<b>MW164863</b>	<b>MW160934</b>
<i>C. mayeri_2</i> – Cebu, Philippines	<b>MW164864</b>	<b>MW160935</b>
<i>C. mayeri_2</i> – Luzon Island, Philippines	<b>MW164866</b>	<b>MW160937</b>
<i>C. mayeri_1</i> – Luzon Island, Philippines	<b>MW164865</b>	<b>MW160936</b>
<i>C. ornata</i> – ?Kamo Aquarium, Indonesia	AB720918	AY319472
<i>C. ornata</i> – Indonesia	–	AY319473
<i>C. xamachana_1</i> – Panama	JN700936	JN700936
<i>C. xamachana_2</i> – Panama	KY610614	KY610559
<i>Cassiopea</i> sp. 1 – Queensland, Australia	–	AY319471
<i>Cassiopea</i> sp. 2_1 – Papua New Guinea	–	MF742198
<i>Cassiopea</i> sp. 2_2 – Papua New Guinea	–	MF742199
<i>Cassiopea</i> sp. 3_1 – Hawaii, USA	–	AY331594
<i>Cassiopea</i> sp. 3_2 – Hawaii, USA	–	AY331595
<i>Cassiopea</i> sp. 4_1 – Palau	MZ366351	LC198739
<i>Cassiopea</i> sp. 4_2 – Palau	MZ366351	LC198740
<i>Cassiopea</i> sp. 6_1 – Palau	–	LC198754
<i>Cassiopea</i> sp. 6_2 – Palau	–	LC198763
<i>Mastigias papua</i>	KY610621	KU901434
<i>Versuriga anadyomene</i>	KX904852	KX904853

**Table S2. Mitochondrial ribosomal gene *16S* rRNA and mitochondrial protein-encoding gene cytochrome *c* oxidase I (*COI*) sequences used for combined-marker analysis without missing data**

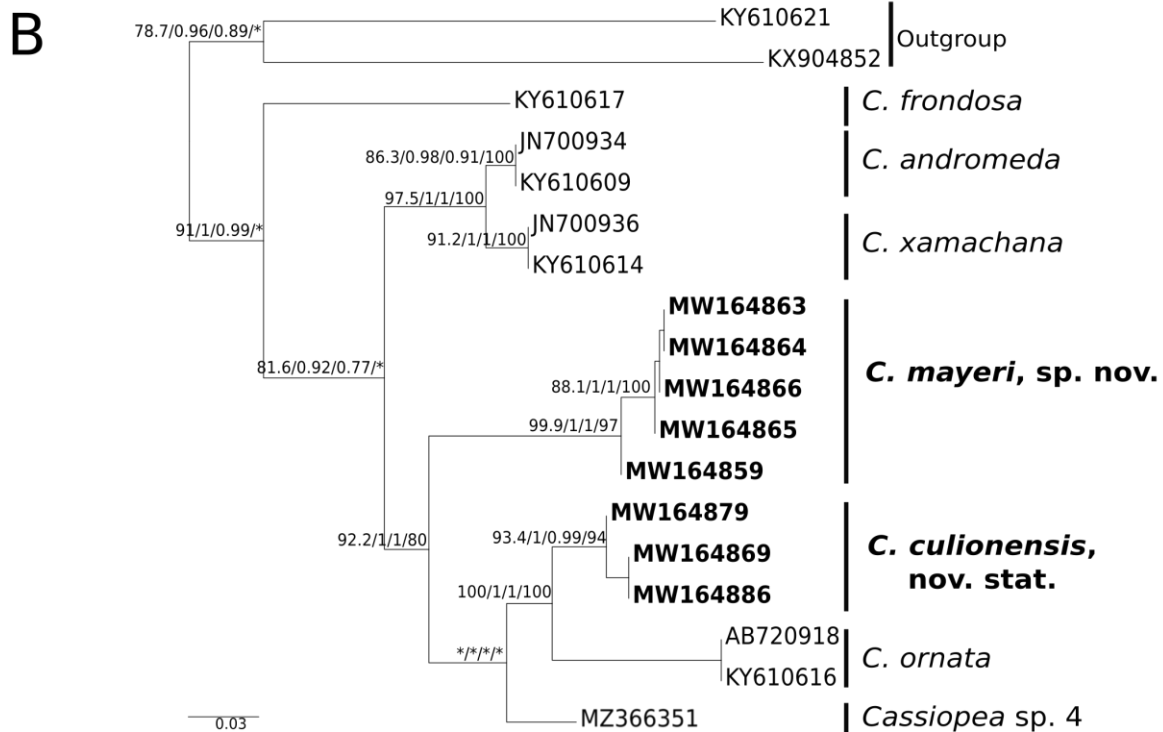
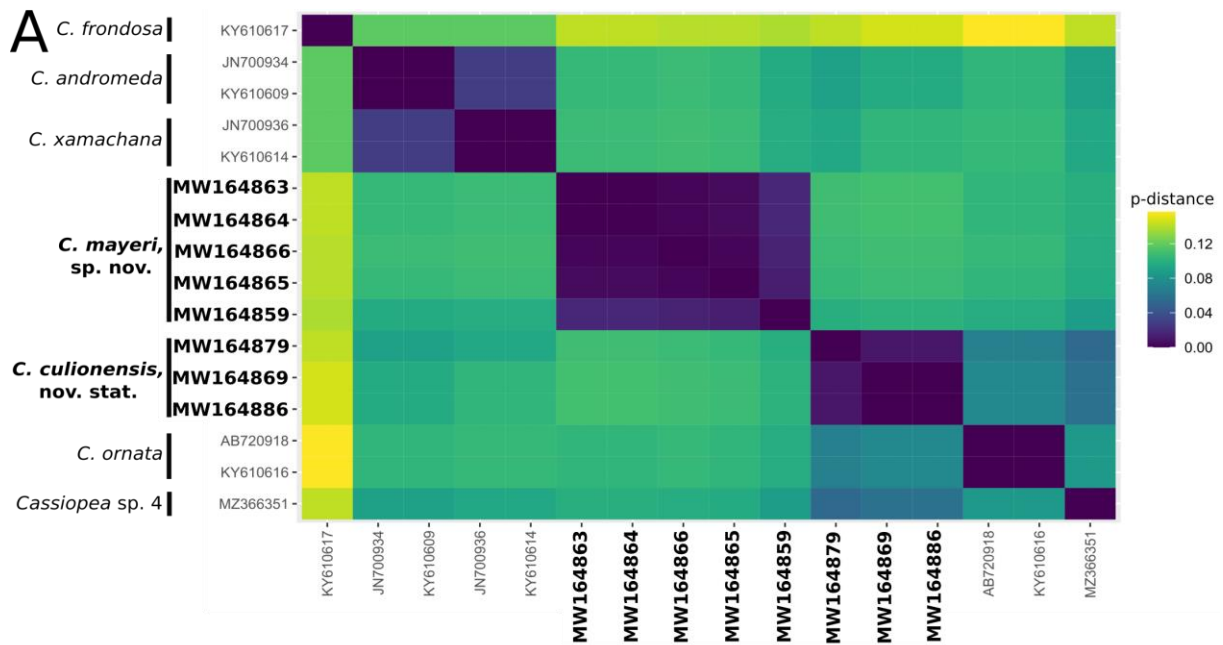
Terminal ID corresponds to terminal names on maximum likelihood tree based on *16S+COI* (Fig. S3)

Terminal ID	<i>16S</i>	<i>COI</i>
<i>Cassiopea andromeda</i> – French Polynesia	JN700934	JN700934
<i>C. andromeda</i> – Mexico	KY610609	KY610551
<i>C. culionensis_1</i> – Cebu, Philippines	<b>MW164879</b>	<b>MW160923</b>
<i>C. culionensis_2</i> – Cebu, Philippines	<b>MW164869</b>	<b>MW160913</b>
<i>C. culionensis_3</i> – Cebu, Philippines	<b>MW164886</b>	<b>MW160930</b>
<i>C. frondosa</i> – Florida, USA	KY610617	AY319467
<i>C. mayeri</i> – Japan	<b>MW164859</b>	<b>MW160931</b>
<i>C. mayeri_1</i> – Cebu, Philippines	<b>MW164863</b>	<b>MW160934</b>
<i>C. mayeri_2</i> – Cebu, Philippines	<b>MW164864</b>	<b>MW160935</b>
<i>C. mayeri_2</i> – Luzon Island, Philippines	<b>MW164866</b>	<b>MW160937</b>
<i>C. mayeri_1</i> – Luzon Island, Philippines	<b>MW164865</b>	<b>MW160936</b>
<i>C. ornata</i> - ?Kamo Aquarium, Indonesia	AB720918	AY319472
<i>C. xamachana_1</i> – Panama	JN700936	JN700936
<i>C. xamachana_2</i> – Panama	KY610614	KY610559
<i>Mastigias papua</i>	KY610621	KU901434
<i>Versuriga anadyomene</i>	KX904852	KX904853

**Table S3. Mitochondrial ribosomal gene 16S rRNA and mitochondrial protein-encoding gene cytochrome *c* oxidase I (*COI*) sequences used for network construction**

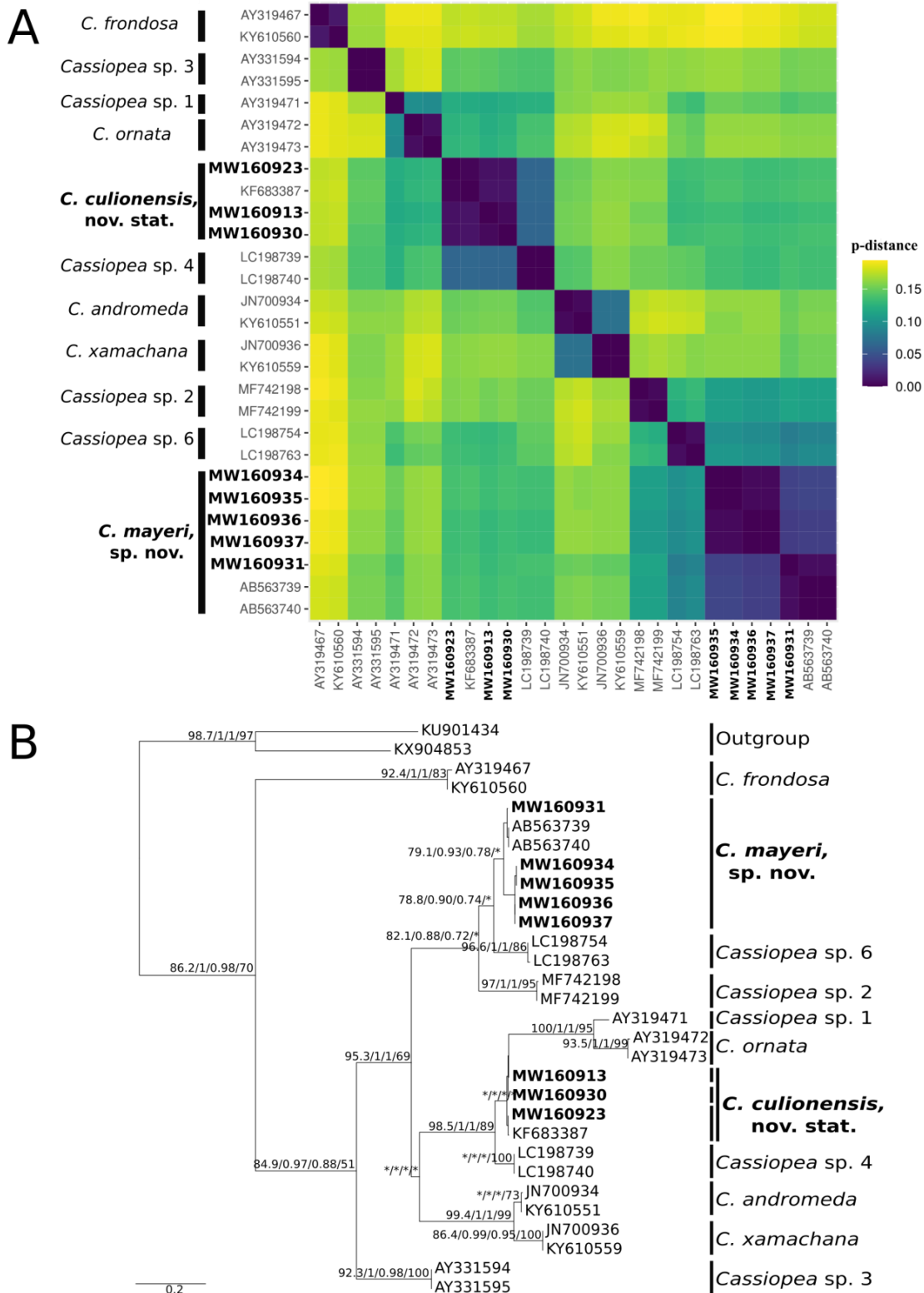
GenBank accession numbers of sequences obtained in this study are underlined. N/A, Not applicable

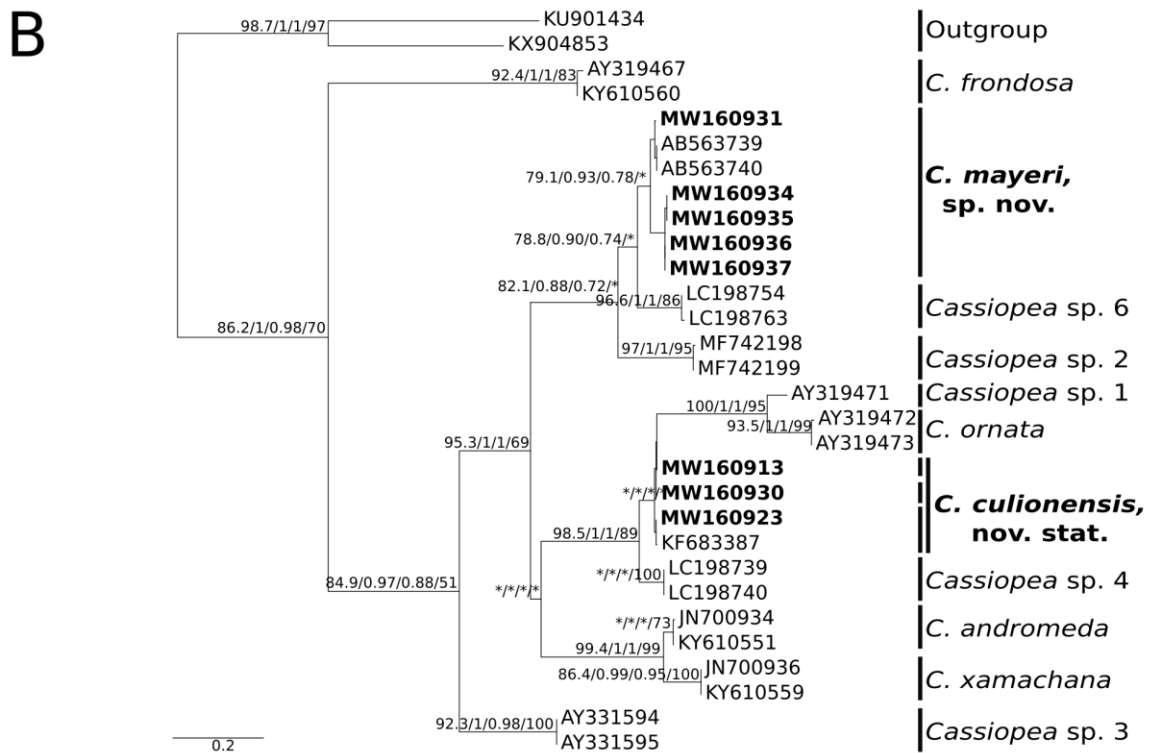
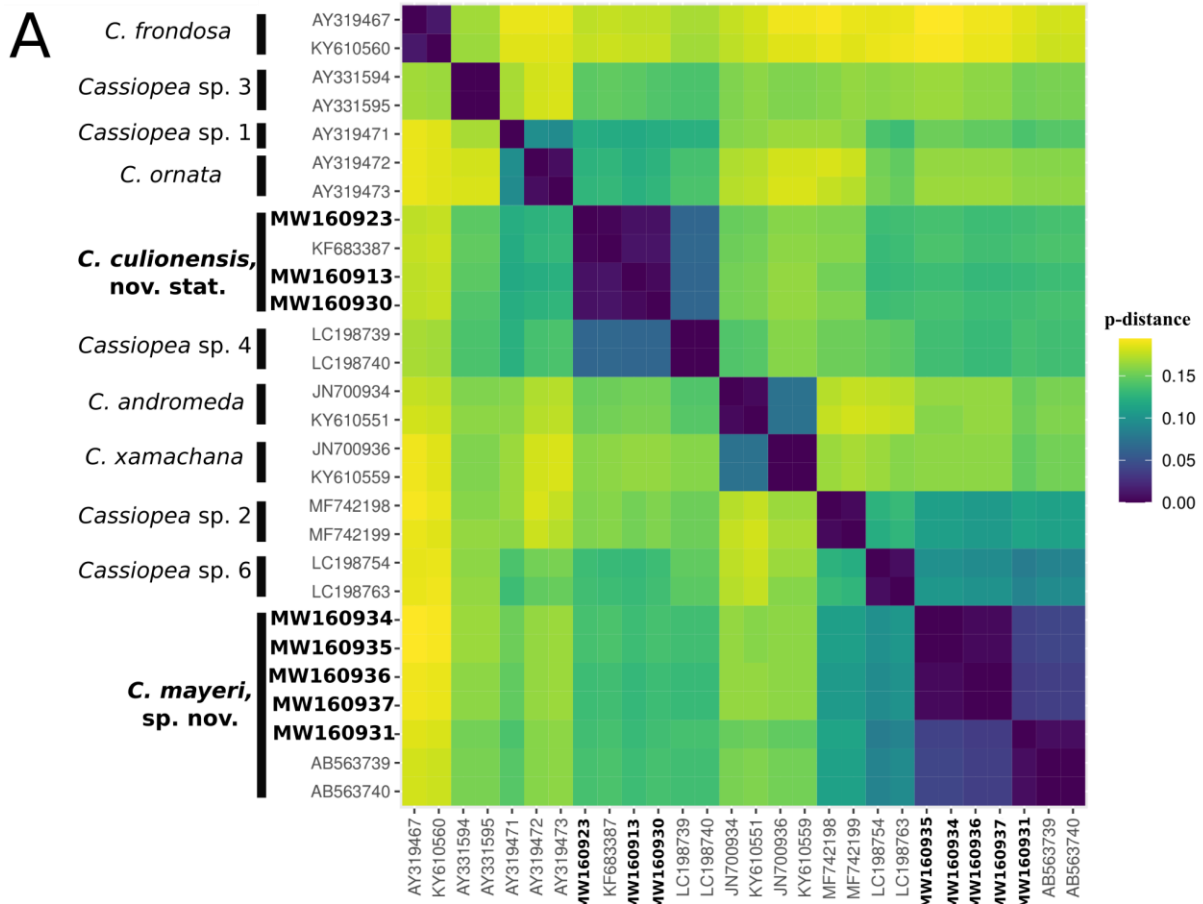
Species	Voucher code	<i>16S</i>	<i>COI</i>
<i>Cassiopea culionensis</i> , stat. nov.	gACM00279	<u>MW164876</u>	<u>MW160920</u>
	gACM00281	<u>MW164877</u>	<u>MW160921</u>
	gACM00282	<u>MW164867</u>	<u>MW160911</u>
	gACM00284	<u>MW164868</u>	<u>MW160912</u>
	gACM00285	<u>MW164869</u>	<u>MW160913</u>
	gACM00286	<u>MW164870</u>	<u>MW160914</u>
	gACM00287	<u>MW164875</u>	<u>MW160919</u>
	gACM00288	<u>MW164871</u>	<u>MW160915</u>
	gACM00290	<u>MW164872</u>	<u>MW160916</u>
	gACM00292	<u>MW164873</u>	<u>MW160917</u>
	gACM00293	<u>MW164874</u>	<u>MW160918</u>
	MZUSP 8632, gACM00340	<u>MW164878</u>	<u>MW160922</u>
	MZUSP 8633, gACM00341	<u>MW164879</u>	<u>MW160923</u>
	MZUSP 8634, gACM00342	<u>MW164880</u>	<u>MW160924</u>
	MZUSP 8635, gACM00343	<u>MW164881</u>	<u>MW160925</u>
	MZUSP 8636, gACM00344	<u>MW164882</u>	<u>MW160926</u>
	MZUSP 8637, gACM00345	<u>MW164883</u>	<u>MW160927</u>
	MZUSP 8638, gACM00346	<u>MW164884</u>	<u>MW160928</u>
	MZUSP 8639, gACM00347	<u>MW164885</u>	<u>MW160929</u>
	MZUSP 8640, gACM00348	<u>MW164886</u>	<u>MW160930</u>
N/A	N/A	KF683387	
<i>C. mayeri</i> , sp. nov.	N/A	N/A	AB563739
	N/A	N/A	AB563740
	FLMNH 007505	<u>MW164859</u>	<u>MW160931</u>
	FLMNH 009664/CASIZ 201000	<u>MW164865</u>	<u>MW160936</u>
	FLMNH 009665/CASIZ 200996	<u>MW164866</u>	<u>MW160937</u>
	gACM00283	<u>MW164860</u>	<u>MW160932</u>
	gACM00289	<u>MW164861</u>	<u>MW160933</u>
	gACM00291	<u>MW164862</u>	N/A
	gACM00280	<u>MW164863</u>	<u>MW160934</u>
	MZUSP 8631, gACM00339	<u>MW164864</u>	<u>MW160935</u>



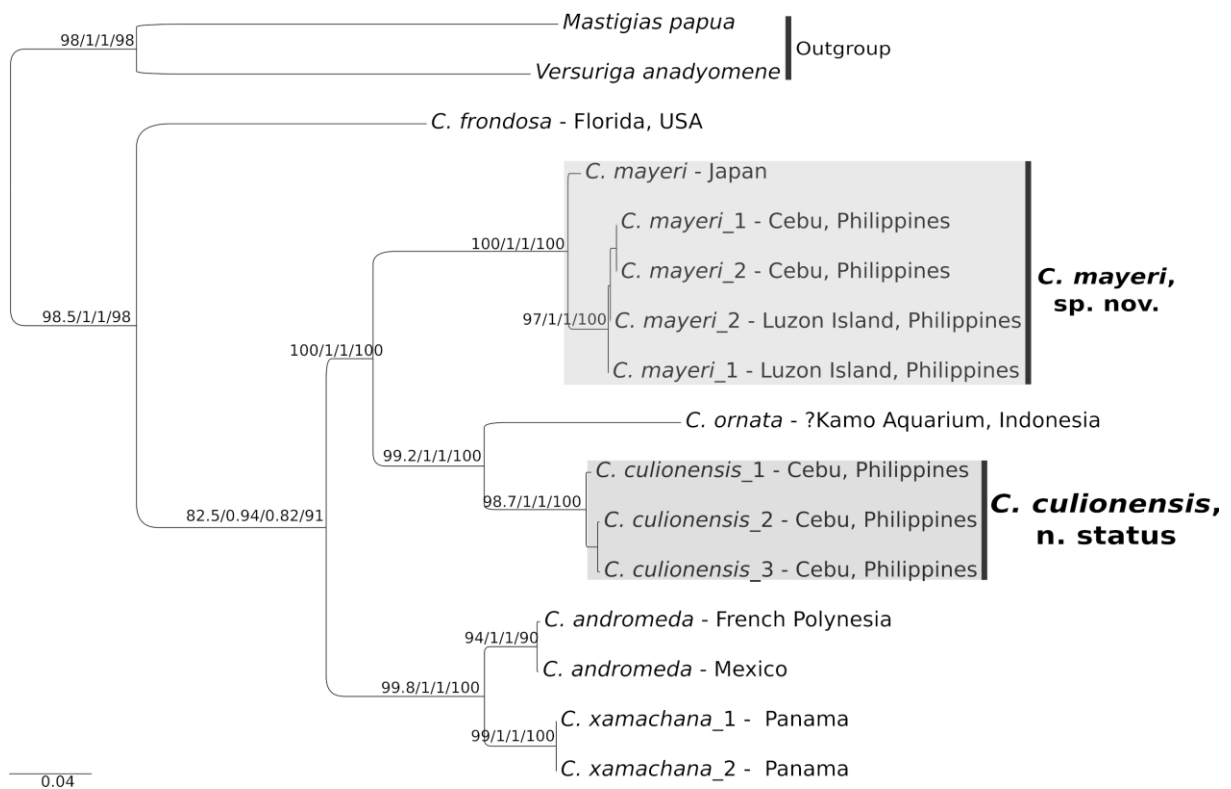
**Fig. S1.** Heatmap of pairwise uncorrected  $p$ -distances and maximum likelihood tree based on mitochondrial ribosomal gene  $16S$  rDNA data. (A) Heatmap of pairwise uncorrected  $p$ -distances. The colours from blue to yellow indicate low to high  $p$ -distance. For taxa names and other information, see the column ‘GenBank’ in Table 1. (B) Maximum likelihood tree based on  $16S$  sequences ( $-\ln L$  2089.353). Clade stability values are shown on branches (as in figure order: Shimodaira–Hasegawa (SH)- approximate likelihood ratio test (aLRT) (%), parametric aLRT/aBayes, bootstrap values (%); Asterisks (\*) indicate less than 0.7, 70). For taxa names and other information, see the column ‘GenBank’ in Table 1.







**Fig. S3.** Maximum likelihood tree based on mitochondrial ribosomal gene *16S* rRNA and mitochondrial protein-encoding gene cytochrome *c* oxidase I (*COI*) data ( $-\ln L$  4322.097). Grey rectangles indicate sequences obtained during this study. Clade stability values are shown on branches (as in figure order: Shimodaira–Hasegawa (SH)- approximate likelihood ratio test (aLRT) (%), parametric aLRT, aBayes, bootstrap values (%)). For taxa names and other information, see Table S2.



**Fig. S4.** *Cassiopea* aff. *mayeri*, photographs of the bigger specimen of the lot USNM 27941. (A) Oral view of the oral disc and arms, which were detached. (B) Detail of the appendages at the oral disc. Black arrows indicate fungiform (peltate) appendages, white arrows indicate tuber-shaped appendages. (C) Appendages of the oral arm. White arrows indicate the remainders of small appendages. (D) Exumbrellar view of a rhopalium and a rhopaliar pit.