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RESEARCH PAPER

# Taxonomy and systematic positions of three new *Callistoctopus* species (Octopoda, Octopodidae) discovered in coastal waters of China

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## Abstract

The genus *Callistoctopus* comprises 13 species, and has been reported mostly in the Western Pacific Ocean. Here, we described three new species from China, *Ca. paucilamellus* sp. nov., *Ca. sparsus* sp. nov., and *Ca. gracilis* sp. nov., based on morphometric and meristic characteristics. The diagnoses, descriptions and detailed morphometric data are provided for each species. The cytochrome c oxidase I (COI) genes of the three new species are sequenced, and compared with related species and analyzed for their systematic positions. Both phylogenetic trees constructed using three mitochondrial genes (12S rRNA, 16S rRNA, COI) and one nuclear gene (Rhodopsin) revealed that our new species formed into two distinct clades with strong support values. One clade included *Ca. gracilis* sp. nov., *Ca.* sp. 1, *Ca. xiaohongxu*, *Ca. tenuipes* and *Ca. paucilamellus* sp. nov., which clustered together. The other clade showed that *Ca. sparsus* sp. nov. was closely related to *Ca.* sp. 2 and *Ca.* sp. 3. *Ca. luteus* and *Ca. macropus* were located at the base of the *Callistoctopus* group. Based on our integrative studies, both morphological and molecular evidence suggested strongly that *O. minor* is more likely to be classified as a species of *Callistoctopus*. Morphological comparisons were made between the three new species and related taxa, which could be recognized based on the 7–8 gill lamellae of each demibranch, numerous small black spots on the subdermal layer of the arms, and an elongated body.

**Keywords** *Callistoctopus* · New species · Coastal waters of China · Species delimitation · Systematic position

## Introduction

Octopods are among the most species-rich and well-known lineages of coleoid cephalopods, with approximately 300 species described, worldwide (Allcock et al. 2015; Sauer et al. 2021). Since the 1990s, considerable research has been focused on octopus systematics, particularly in the Australian regions and Indo-West Pacific, which includes Southeast Asia, defined herein as the East China Sea to the South China Sea and the Andaman Sea, a region well known in terms of systematics and natural history (Ibáñez et al. 2021; Kubodera and Lu 2002).

The genus *Callistoctopus* comprises 13 species that inhabit shallow waters in all oceans, except the polar regions. They are particularly abundant in the tropical Indo-Pacific region and usually inhabit continental shelves down to approximately 200 m (Norman et al. 2013). New species of the genus *Callistoctopus* have mainly been reported in the twentieth century (Norman 1993; Norman and Sweeney 1997; Sasaki 1929; Stranks 1990; Voss 1979). After that, no reports were published until 2022, when two consecutive reports identified additional species within the *Callistoctopus* genus in coastal waters of China (Li et al. 2022; Zheng et al. 2022). These findings suggest the presence of substantial unexplored diversity in the geographical area sampled, and emphasize the need for further research on this genus.

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**Special Topic:** Fishery Science and Technology.

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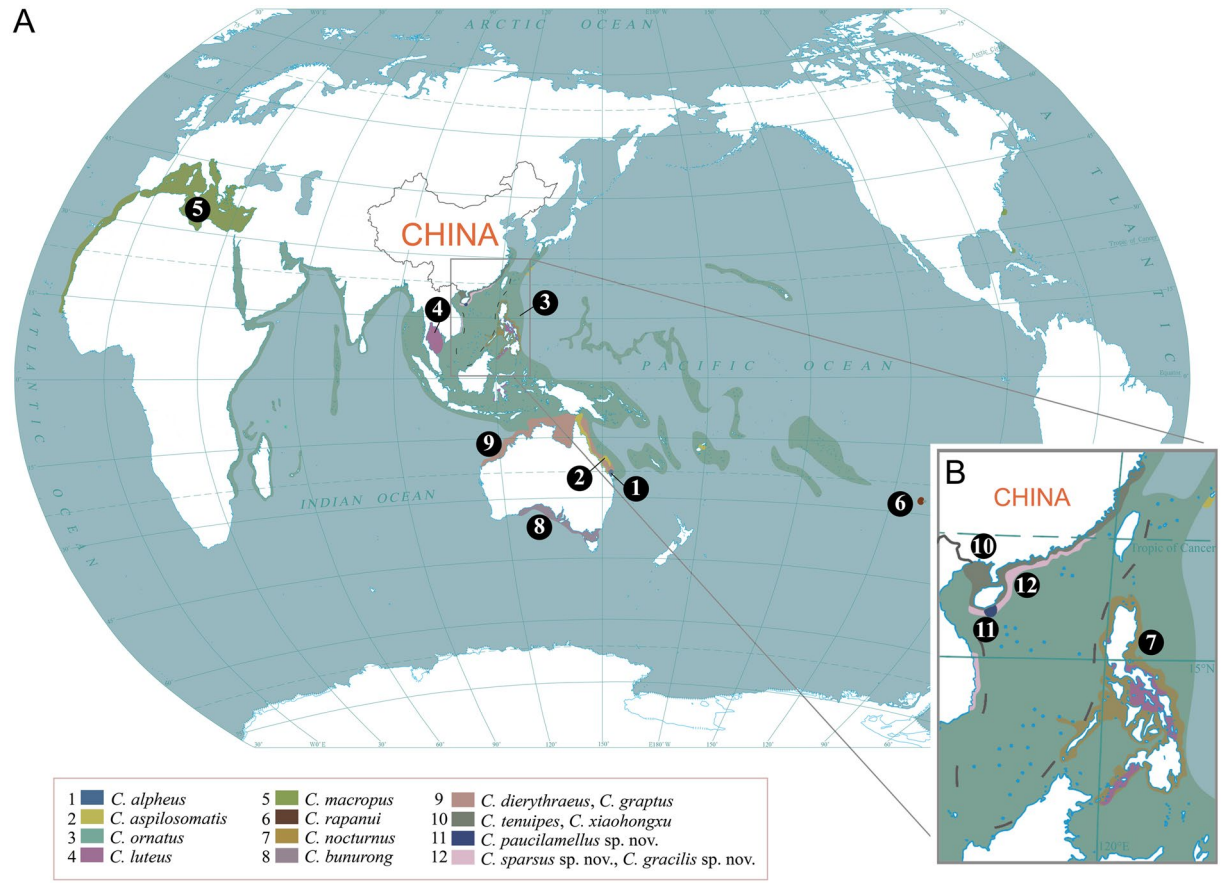


**Fig. 1** Known distribution of *Callistoctopus* species. **A** World map with different colors indicating different species **B** Enlarged view of the sampling and surrounding sea area

The taxonomy of most taxa is challenging due to the morphological similarities and phenotypic flexibility observed in octopuses, and the genus *Callistoctopus* is no exception (Acosta-Jofré et al. 2012). Previously, members of this genus have been labelled under the name "*Octopus macropus* group" (e.g., Norman 1993, 2000). Many individuals in this group were mistakenly identified as *Octopus macropus* (= *Callistoctopus macropus*). While efforts have been made to distinguish most species within this group and classify them as distinct species of the genus *Callistoctopus*, problems remain with the taxonomy. One major issue is the incomplete morphological information available, particularly for species including *Ca. macropus* and *Ca. lechenaultii*. The lack of detailed descriptions of key structures, such as the mantle, funnel and hectocotylized arm has hindered a comprehensive understanding of the morphology of the genus *Callistoctopus*, leading to confusion between species within this genus. Additionally, there is a lack of sufficient molecular data, especially for the cytochrome oxidase subunit 1 (COI) gene, which is commonly used in octopod classification. Therefore, many species lack molecular data, and few studies have combined morphological and phylogenetic results to investigate the phylogenetic relationships within or between genera.

In the present study, three new *Callistoctopus* species collected from the coastal waters of China (Fig. 1) are described. They are placed within the genus *Callistoctopus* based on morphological and DNA sequencing data. Moreover, this study used the data to address the phylogenetic relationships within *Callistoctopus*, compared the characteristics of related species of the genus *Callistoctopus* with those of our new species, and summarized the key to species of the genus *Callistoctopus*.

## Materials and methods

### Morphological analyses

All specimens were collected from the East China Sea or South China Sea, as detailed Fig. 1. Muscle tissue was sampled from the mantle and stored in 100% ethyl alcohol until subsequent DNA analysis. The specimens were fixed in 10% buffered formalin for one week before transfer to

75% ethyl alcohol for permanent storage to determine morphological analysis. Specimens were deposited at the Genetics and Breeding Science of Shellfish (GBSS), Fisheries College, Ocean University of China (OUC), Qingdao, China.

Morphometric and meristic characteristics were measured using either a ruler (to the nearest millimeter) or dial calipers (precision of 0.1 mm), following Roper and Voss (1983) and Norman and Sweeney (1997). Twenty-seven indices of morphological measurements and counts were used in the descriptions, and are shown in Supplementary Table S1, where the arms are numbered (1–4) starting from the dorsal pair, and web sectors are designated by letters (A–E), starting from the dorsal web. For scalation measurements, characteristics were observed using a Nikon SMZ1270 binocular stereomicroscope and measured using NIS-Elements Microscope Imaging Software v4.60.00 (Nikon).

The beaks and radulae were extracted from the buccal masses of some specimens, cleaned and illustrated. The radulae were cleaned with 7% NaOH, critical-point dried and coated with gold before scanning using a VEGA3 scanning electron microscope (TESCAN, Brno, Czech Republic). Beaks were cleaned and stored in 75% ethyl alcohol.

## DNA extraction, amplification, and sequencing

Total genomic DNA from each sample was extracted using the E.Z.N.A. Mollusk DNA Kit (OMEGA Bio-Tek Co., Norcross, GA, USA), following the manufacturer's instructions. The concentration and quality of the DNA were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and agarose gel electrophoresis. Fragments of two mitochondrial (Cytochrome oxidase subunit I, COI and 16S ribosomal RNA, 16S) and one nuclear (Rhodopsin) gene were amplified in this study (Allcock et al. 2008; Folmer et al. 1994). The 12S ribosomal RNA (12S) and partial gene fragments of some species were extracted from our unpublished mitochondrial genome (see Supplementary Table S2). The polymerase chain reaction (PCR) amplification was carried out in 50 μL PCR reaction consisting of 25 μL 2 × *Taq* Plus Master Mix II (Vazyme Biotech, Nanjing, China), 2 μL of each primer (20 mmol/L), 0.2–1 μL of non-diluted DNA template, and 20–20.8 μL of sterile distilled water (adjusted to DNA extract). The PCR conditions for amplification were as follows: initial denaturation at 94 °C for 3 min; 32 cycles at 94 °C for 45 s, annealing temperature at 50 °C for 1 min, and 72 °C for 1 min 20 s; and a final 5 min at 72 °C. The PCR products were confirmed by 1.5% agarose gel electrophoresis, purified using the EZ Spin Column PCR Product Purification Kit (Tiangen Biotech, Beijing, China), and sequenced at the Sangon Biotech Company (Shanghai, China) using an ABI 3730 XL automatic sequencer (Applied Biosystems, NY, USA) and the primer walking method. COI gene sequence comparison of the three new species and other related taxa was performed using BioEdit version 7.2.5 (Hall 1999) (Supplementary Table S2). The intraspecific and intergeneric genetic distances were calculated by Kimura 2-parameter (K2P) model (Kimura 1980) using MEGA v.6.0 (Tamura et al. 2013).

## Molecular data and phylogenetic analyses

All DNA sequences were assembled and adjusted manually using the SeqMan v.7.1.0 (DNASTAR software package) and BioEdit v.7.2.5 (Hall 1999), and then aligned using Clustal W (Thompson et al. 1994) with default settings in MEGA v.6.0 (Tamura et al. 2013). Dubiously aligned nucleotide positions were omitted using Gblocks v. 0.91b (Castresana 2000; Talavera and Castresana 2007) with the default settings. All loci were concatenated with FASconCAT v1.0.pl (Kück and Meusemann 2010). Phylogenetic analyses were performed using Bayesian inference (BI) and maximum likelihood (ML) methods. The PartitionFinder (Kalyaanamoorthy et al. 2017) plugin integrated into PhyloSuite v1.2.1 (Zhang et al. 2020) was used to find the best-fit partitioning schemes and models using a search with Akaike information criterion (AICc). The GTR + I + G model was estimated as the best model for each gene sequence. BI analyses were performed using MrBayes v3.2.6 (Ronquist et al. 2012), with 2,000,000 generations using the Markov chain Monte Carlo (MCMC) command. Sampling was performed every 100 generations, and the first 25% of the sampled data were discarded as burn-in. Tracer v1.7 (Rambaut et al. 2018) was used to check the convergence based on the effective sampling size (ESS > 200) and the parameter distribution of the chains. The ML trees were inferred using IQ-TREE v1.6.12 (Nguyen et al. 2015), with the auto-model selection mode for 100,000 ultra-fast bootstrap replicates. All phylogenetic results were visualized using FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree).

## Species delimitation

Putative species were determined by applying automatic barcode gap discovery (ABGD) (Puillandre et al. 2012), assembly species by automatic partitioning (ASAP) (Puillandre et al. 2021), and Bayesian implementation of the Poisson tree processes (bPTP) (Zhang et al. 2013) to our dataset. ABGD was performed using a web server (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) with

the Kimura-2-parameter (K2P) model. COI gene alignments were used as the input files. All analyses were performed with the following parameters: Pmin = 0.0008, Pmax = 0.007, steps = 50, X = 1, and Nb bins = 20. ASAP was performed using a web server (https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html) with default settings. bPTP was implemented on the bPTP web server (http://species.h-its.org/). Rooted trees from the ML and BI analyses were input with 200,000 Markov chain MCMC generations and other default parameters.

## Results

### ZooBank registration

This article: urn:lsid:zoobank.org:pub:55E7928A-D776-4181-87D2-C644B1BF80FD.

*Callistoctopus paucilamellus* sp. nov.: urn:lsid:zoobank.org:act:E5A7CEC2-8465-4F0D-8423-5483BA75C54F.

*Callistoctopus sparsus* sp. nov.: urn:lsid:zoobank.org:act:AE0ED067-D6B6-4E42-BA12-2B5D520A55C2.

*Callistoctopus gracilis* sp. nov.: urn:lsid:zoobank.org:act:75533119-0DFA-4647-85F2-37882789C453.

### Taxonomy

Class: Cephalopoda Cuvier, 1795.

Order: Octopoda Leach, 1818.

Suborder: Incirrata Grimpe, 1916.

Family: Octopodidae d'Orbigny, 1840.

Genus: *Callistoctopus* Taki, 1964.

*Callistoctopus* Taki, 1964

Type species: *Callistoctopus arakawai* Taki, 1964 [= *Ca. ornatus* (Gould 1852)].

The genus *Callistoctopus* was first proposed in 1964 by Taki, who described it as a group of large animals with a robust and stout body structure, akin to *Octopus*. However, this description was based on two species, *Ca. arakawai* and *Ca. magnocellatus*, one of which was later recognized as *O. cyanea*. Norman et al. (2013) summarized the genus in a FAO species catalogue, based on the morphology of nine published species of *Callistoctopus*. They included descriptions of additional characters, such as arm formula, web formula. Since then, and including the present study, five new species of the *Callistoctopus* have been reported, which require the addition of certain morphological characters (e.g., funnel organ shape and number of gill lamellae) to the genus *Callistoctopus*. Therefore, this study presents an amended diagnosis based on new information and previous reports.

Amended diagnosis (adapted from Norman et al. 2013): Small to large species (dorsal mantle length (DML) 27.5–190.8 mm). Mantle ovoid to elongated cylindrical. Stylets reduced or absent. Arms muscular and long, 3 to 8 times mantle length. Dorsal arms always longest (1 > 2 > 3 > 4 or 1 > 2 > 4 > 3). Arm autotomy at distinct plane absent. Web depths shallow to moderate, ~ 5–20% of longest arm length. Dorsal webs always deepest (typically A > B > C > D > E). Interbrachial web pouches absent. Suckers in two rows. Enlarged suckers present in mature males of a few member species. Funnel organ W-, UU- or \/\-shaped, large. Gills with 7–15 lamellae per outer demibranch. Radula with 9 elements, 7 rows of teeth plus marginal plates. Rachidian tooth with 2–3 lateral cusps and very tall mesocone. Posterior salivary glands moderate to large, equal to or larger than buccal mass. Distinct crop present as side-branch off oesophagus. Ink sac present. Anal flaps present. Third right arm of male hectocotylized, distinctly shorter than opposite arm. Ligula and calamus present; ligula cylindrical with deep groove. Spermatophores small, unarmed. Eggs small to large. Color typically red-brown to red with white spots or bars on mantle, head and arms. False eye-spots (ocelli) absent. Skin smooth or with scattered low papillae, patch and groove system not evident. Conspicuous primary papillae present over each eye. Continuous skin ridge around lateral margin of mantle absent.

### *Callistoctopus paucilamellus* sp. nov. (Fig. 2; Supplementary Tables S3, S4)

#### Type and voucher materials

*Holotype*. OUC-202104100301, Sanya, Hainan, China, 18°22′ N, 109°7′ E, 10 April 2021, mature male, 54.0 mm DML, coll. GBSS.

*Paratypes*. OUC-202104100302, Sanya, Hainan, China, 18°22′ N, 109°7′ E, 10 April 2021, mature female, 65.1 mm DML, coll. GBSS. OUC-202104140306, Lingshui, Hainan, China, 18°25′ N, 109°59′ E, 14 April 2021, immature female, 46.0 mm DML, coll. GBSS. OUC-202104140309, Lingshui, Hainan, China, 18°25′ N, 109°59′ E, 14 April 2021, immature female, 49.4 mm DML, coll. GBSS. OUC-202104140310, Lingshui, Hainan, China, 18°25′ N, 109°59′ E, 14 April 2021, immature female, 46.4 mm DML, coll. GBSS. OUC-202104140312, Lingshui, Hainan, China, 18°25′ N, 109°59′ E, 14 April 2021, immature female, 47.1 mm DML, coll. GBSS. OUC-202104140317, Lingshui, Hainan, China, 18°25′ N, 109°59′ E, 14 April 2021, immature female, 51.6 mm DML, coll. GBSS. OUC-202104140319, Lingshui, Hainan, China, 18°25′ N, 109°59′ E, 14 April 2021, submature male, 38.5 mm DML, coll. GBSS. OUC-202104140328, Lingshui, Hainan, China, 18°25′ N, 109°59′ E, 14 April 2021, submature

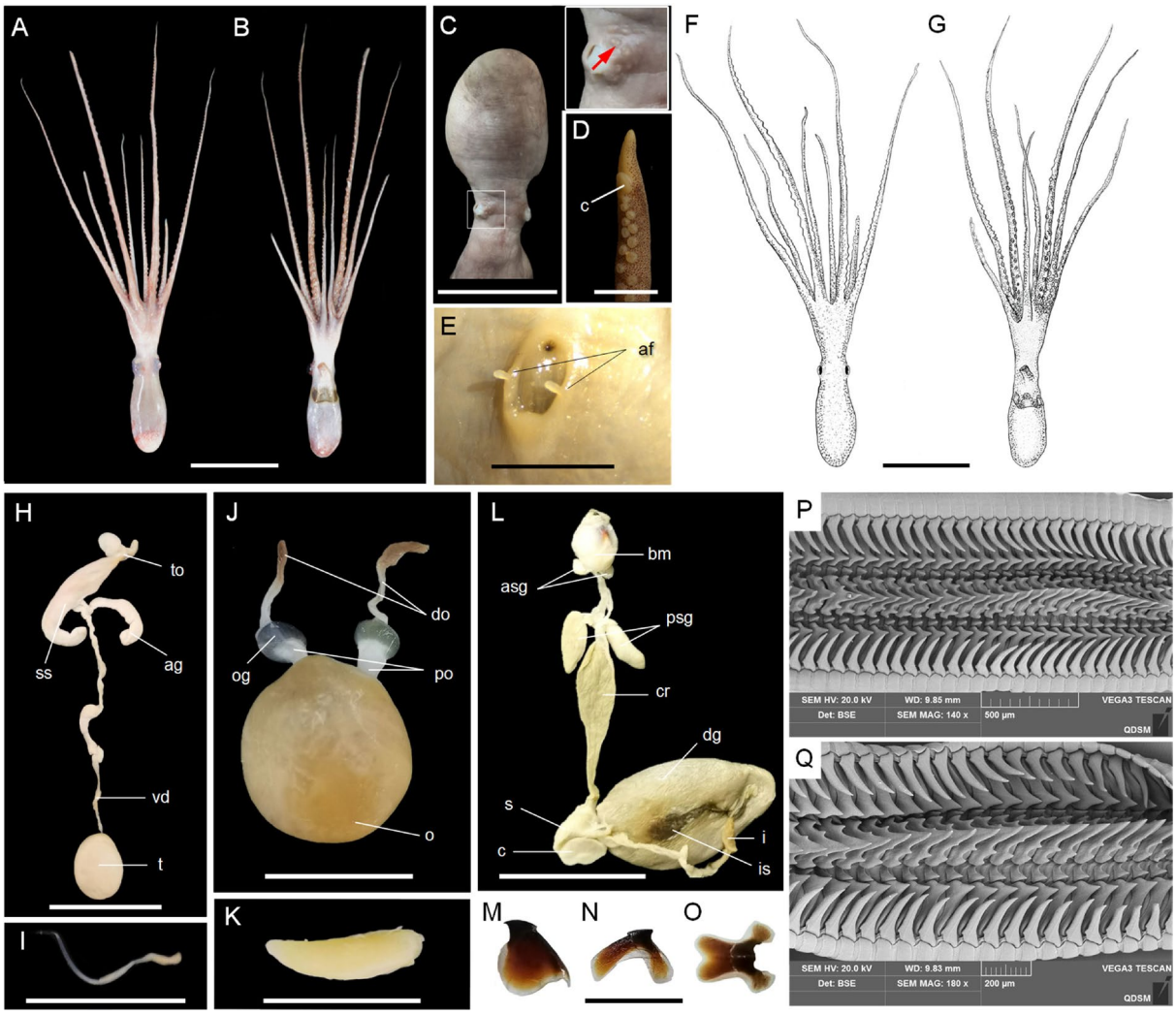


**Fig. 2** *Callistoctopus paucilamellus* sp. nov. from alcohol-fixed specimens. **A** Dorsal view (OUC-202104140336). **B** Ventral view (OUC-202104140336). **C** Dorsal view of partial body (OUC-202104100302), arrow shows the tiny bumps. **D** Hectocotylus of mature male (OUC-202104140329). **E** Anus (OUC-202104100302). **F**, **G** Line drawings of dorsal (**F**) and ventral (**G**) views of fixed specimens. **H** Male reproductive system (OUC-202104100301). **I** Spermatophore (OUC-202104100301). **J** Female reproductive system (OUC-202104100303). **K** Single laid egg (OUC-202104100303). **L** Whole view of digestive system (OUC-202104100303). **M**–**O** Lateral (**M**) view of upper beak (OUC-202104140303), lateral (**N**) and top (**O**) views of lower beak (OUC-202104140303). **P** Radula of male specimen (OUC-202104100301). **Q** Radula of female specimen (OUC-202104140336). *af* anal flaps, *ag* accessory gland, *asg* anterior salivary gland, *bm* buccal mass, *c* caecum, *cr* crop, *dg* digestive gland, *do* distal oviduct, *i* intestine, *is* ink sac, *o* ovary, *og* oviducal gland, *po* proximal oviduct, *psg* posterior salivary gland, *s* stomach, *ss* spermatophore storage sac, *t* testis, *to* terminal organ, *vd* vas deferens. Scale bars: **A**, **B**, **C**, **F**, **G** = 50 mm, **D**, **E** = 2 mm, **H**, **I**, **J**, **L** = 20 mm, **K** = 10 mm, **M**, **N**, **O** = 5 mm

male, 41.0 mm DML, coll. GBSS. OUC-202104140329, Lingshui, Hainan, China, 18°25′ N, 109°59′ E, 14 April 2021, submature male, 37.3 mm DML, coll. GBSS.

*Other materials*. OUC-202104100303, Sanya, Hainan, China, 18°22′ N, 109°7′ E, 10 April 2021, mature female, 54.1 mm DML, coll. GBSS. OUC-202104140302, Lingshui, Hainan, China, 18°25′ N, 109°59′ E, 14 April 2021, immature female, 43.0 mm DML, coll. GBSS. OUC-202104140305, Lingshui, Hainan, China, 18°25′ N, 109°59′ E, 14 April 2021, immature female, 47.0 mm DML, coll. GBSS. OUC-202104140311, Lingshui, Hainan, China, 18°25′N, 109°59′ E, 14 April 2021, immature female, 44.9 mm DML, coll. GBSS. OUC-202104140313, Lingshui, Hainan, China, 18°25′ N, 109°59′ E, 14 April 2021, immature female, 41.1 mm DML, coll. GBSS. OUC-202104140314, Lingshui, Hainan, China, 18°25′ N, 109°59′ E, 14 April 2021, immature female, 43.6 mm DML, coll. GBSS.

*Etymology:* Species epithet *paucilamellus* is derived from Latin *paucus*, meaning ‘few’, and Latin *lamellarum*, meaning ‘thin plates’. The name denotes the small number of gill

lamellae (Min. 7) in this species, which is different from that of all other congeners (8–14).

*Diagnosis:* Small to medium-sized species (DML 37.3–65.1 mm, total weight (TW) 6.4–32.8 g). Mantle ovoid to elongated. Arms slender and moderate in length, approximately three to four times mantle length. Dorsal arms longest; arm formula mostly 1 > 2 > 3 > 4. Arm suckers biserial and small. Enlarged suckers absent in both sexes. Webs of moderate depth, webs between dorsal arms deepest (typically A > B > C > D > E), deepest 10.5–14.9% of arm length. Funnel of moderate length, free funnel moderate (29.4–52.8% funnel length). Gills with 7–8 lamellae per demibranch. Mature ovarian eggs very large with low numbers (approximately 49). Right third arm of males hectocotylized with 63–65 suckers. Ligula small, 3.3–5.5% of arm length. Calamus of moderate size, 36.4–37.5% of ligula length. Spermatophores approximately 11. Fresh and fixed specimens light red to grey in color. Skin smooth, with numerous small white spots over dorsal surfaces.

*Description:* The following description is based on 4 males (1 mature and 3 submature) and 11 females (one mature and 10 immature): Some characteristics were described on the basis of partial individuals (e.g., total length (TL) and arm length (AL), which were described on the basis of individuals with intact arms). Measurements and counts are summarized in Supplementary Tables S3 and S4.

### External features

Small to medium-sized species (TL 174.9–218.3–274.9; DML 37.3–46.4–65.1; TWg 6.4–12.5–32.8). Mantle elongate, dorsally longer than wide (mantle width index (MWI) 57.0–66.4–77.0). Head narrower than mantle (head width index (HWI) 29.1–34.6–41.8; head mantle width index (HMWI) 41.8–52.3–63.8) (Fig. 2A, B). Eyes prominent and large (eye length index (ELI) 9.1–10.6–12.7) (Fig. 2A, C). Funnel moderate, with a moderate-sized free funnel (funnel length index (FLI) 28.0–34.5–43.9; free funnel index (FFI) 29.4–43.6–52.8) (Fig. 2B). Funnel organ unknown. Arms moderate in length (approximately 3–4 times mantle length), and slender (arm width index (AWI) 11.2–14.0–16.3) and tapering to tips. Dorsal arms longest; arm formula mostly 1 > 2 > 3 > 4. Webs moderate (web depth index (WDI) 10.5–12.9–14.9), ventral webs shallowest and dorsal webs deepest. Arm suckers biserial and small (normal sucker diameter index (NSDI) 3.7–4.4–4.9). Enlarged suckers absent in mature males. Right third arm of mature males hectocotylized, distinctly shorter than opposite arm (opposite arm index (OAI) 51.8–58.4–61.8). Sucker counts 63–65 on hectocotylus of mature male specimens. Ligula small to moderate (ligula length index (LLI) 3.3–4.6–5.5) with a conspicuous conical calamus (calamus length index (CaLI) 36.4–37.1–37.5) (Fig. 2D). Gills with 7–8 lamellae per demibranch. Fresh and fixed specimens light red to grey in color. Skin smooth with numerous small white spots on dorsal surfaces. Several small bumps present over each eye (Fig. 2C).

*Reproductive system:* Male reproductive system illustrated in Fig. 2H. Male terminal organ hollow tube, thick, diverticulum muscular (terminal organ length index (TOLI) 9.5–10.6–11.4), curved with swollen diverticulum. Vas deferens short. Testis moderate in size. Spermatophore storage sac moderate in length, full of mature spermatophores (spermatophore number (SpN) 11 in storage sac of examined mature male). Spermatophores moderate in length (spermatophore length index (SpLI) 35.9–38.1) (Fig. 2I).

Female reproductive system illustrated in Fig. 2J. Mature ovary oval. Proximal oviducts short and slightly swollen. Distal oviducts short to moderate. Mature ovarian eggs very large, around 7.9–13.3 mm (egg length index (EgLI) 12.1–20.4; egg width index (EgWI) 3.5–6.5) (Fig. 2K). Approximately 49 mature eggs present in examined mature female.

*Digestive system:* Digestive system illustrated in Fig. 2L. Buccal mass large. Anterior salivary glands small, about one quarter of buccal mass. Posterior salivary glands large and long, slightly larger than buccal mass length. Caecum with one whorl. Intestine long with moderate anus. Anal flaps present, elongated (Fig. 2E). Digestive gland well developed. Ink sac distinct and narrow, embedded in surface of digestive gland (Fig. 2L).

Chitinous beaks dark brown. Upper beak (Fig. 2M) with a short-hooked rostrum, slightly broad hood, short wings and broad lateral wall. Lower beak with a short rostrum, narrow hood, long and moderately broad wings (Fig. 2N, O). Radulae with seven transverse rows of teeth and two rows of marginal plates (Fig. 2P, Q). Three lateral teeth flanked on either side of one central rachidian tooth, i.e., a 3·1·3 formula. Rachidian tooth multi-cuspid, and with 2–3 sharp lateral cusps on each side of median cone. Lateral cusps migrating from lateral to medial position over two rows, every four forming a repeating unit. First lateral tooth smallest, with one medial cusp, second lateral tooth with wide heel, one dagger-like cusp, base concave, third lateral tooth with a long blunt cusp, short base.

*Geographical distribution:* This species is known only from the South China Sea near Hainan.

*Remarks*: Before this study, no research has addressed the possibility of the species being unidentified or a potential new species. This newly discovered species *Ca. paucilamellus* sp. nov. overlaps in distribution with *Ca. xiaohongxu* and *Ca. tenuipes* off the coast of China. This new species exhibits morphological similarities to its two congeners by having a low number of gill lamellae per demibranch. However, it may be easily distinguished from them by its body size (approximately 30–60 mm in mantle length, 200–300 mm in total length), body color (red to reddish-brown), smooth skin, large and numerous eggs, and fewer gill lamellae per demibranch (7–8).

## *Callistoctopus sparsus* sp. nov. (Fig. 3; Supplementary Tables S5 and S6)

### Type and voucher materials

*Holotype*. OUC-202106090309, Zhangzhou, Fujian, China, 23°19′ N, 117°23′ E, 9 June 2021, mature male, 50.2 mm DML, coll. GBSS.

*Paratypes*. OUC-202103200301, Lingshui, Hainan, China, 18°25′ N, 109°59′ E, 20 March 2021, mature female, 76.4 mm DML, coll. GBSS. OUC-202103200302, Lingshui, Hainan, China, 18°25′ N, 109°59′ E, 20 March 2021, mature male, 58.5 mm DML, coll. GBSS. OUC-202103200304, Lingshui, Hainan, China, 18°25′ N, 109°59′ E, 20 March 2021, mature female, 63.9 mm DML, coll. GBSS. OUC-202106090304, Zhangzhou, Fujian, China, 23°19′ N, 117°23′ E, 9 June 2021, mature female, 61.1 mm DML, coll. GBSS. OUC-202106090306, Zhangzhou, Fujian, China, 23°19′ N, 117°23′ E, 9 June 2021, mature male, 52.6 mm DML, coll. GBSS. OUC-202106090308, Zhangzhou, Fujian, China, 23°19′ N,

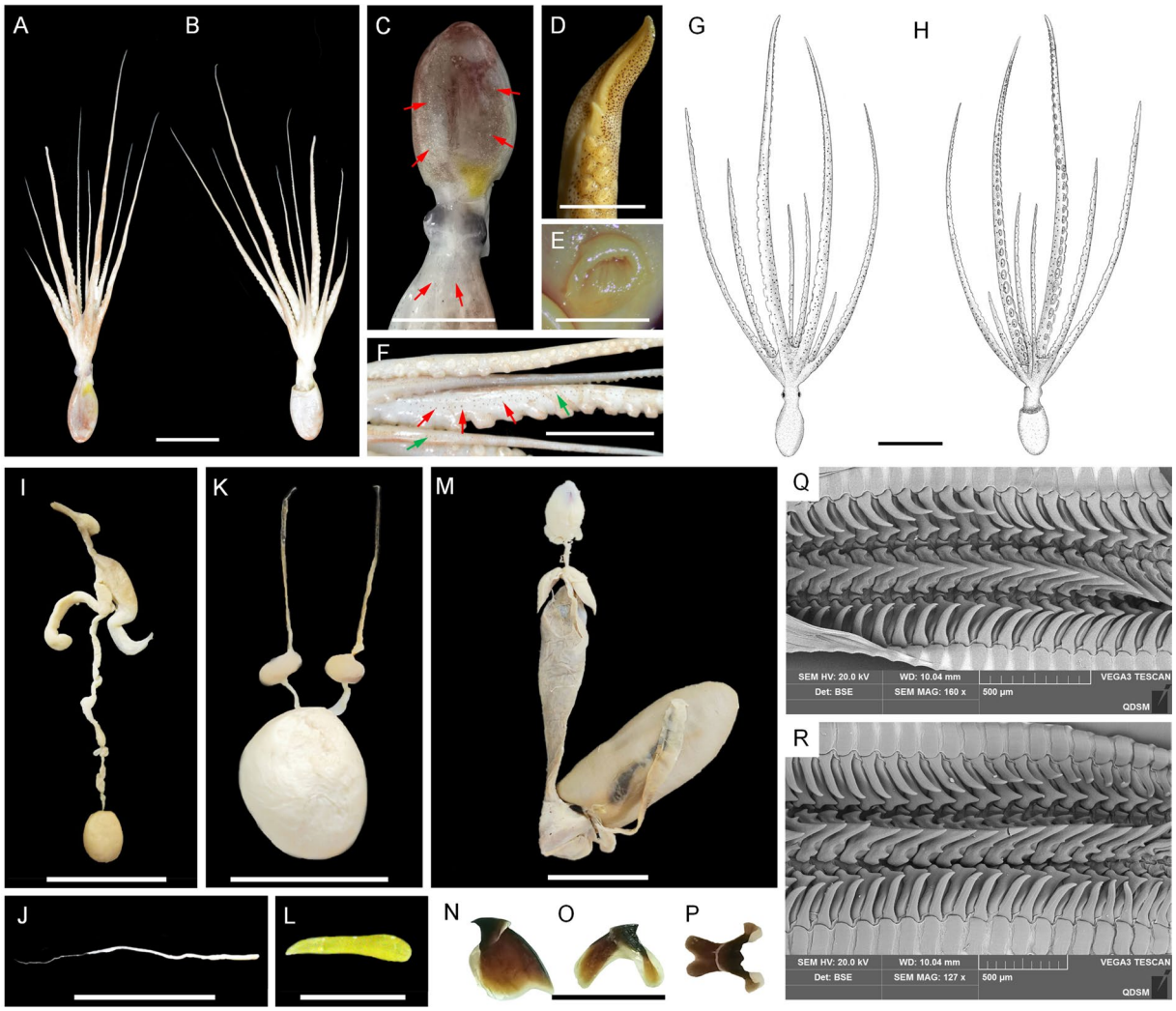


**Fig. 3** *Callistoctopus sparsus* sp. nov. from alcohol-fixed specimens. **A** Dorsal view (OUC-202106090307). **B** Ventral view (OUC-202106090307). **C** Dorsal view of partial body (OUC-202106090307), arrows show the white spots over dorsal surfaces of mantle and bases of arms. **D** Hectocotylus of mature male (OUC-202103200302). **E** Anus (OUC-201812040310). **F** Dorsal surface of arms, arrows show the chromatophores scattered under (red arrows) and over (green arrows) the skin on dorsal surface of arms. **G**, **H** Line drawings of dorsal (**G**) and ventral (**H**) views of fixed specimens. **I** Male reproductive system (OUC-202103200302). **J** Spermatophore (OUC-202103200302). **K** Female reproductive system (OUC-202103200304). **L** Single laid egg (OUC-202103200304). **M** Whole view of digestive system (OUC-202103200304). **N**–**P** Lateral (**N**) view of upper beak (OUC-202104140340), lateral (**O**) and top (**P**) views of lower beak (OUC-202104140340). **Q** Radula of male specimen (OUC-202104140340). **R** Radula of female specimen (OUC-202103200301). Scale bars: **A**, **B**, **G**, **H** = 50 mm, **C**, **F** = 40 mm, **D**, **E** = 2 mm, **I**, **J**, **K**, **M** = 20 mm, **L** = 1 mm, **N**, **O**, **P** = 5 mm

117°23′ E, 9 June 2021, mature female, 74.6 mm DML, coll. GBSS. OUC-202106090310, Zhangzhou, Fujian, China, 23°19′ N, 117°23′ E, 9 June 2021, mature male, 55.8 mm DML, coll. GBSS.

*Other materials*. OUC-201904040301, Zhangzhou, Fujian, China, 23°19′ N, 117°23′ E, 4 April 2019, submature male, 43.4 mm DML, coll. GBSS. OUC-201911260304, Yangjiang, Guangdong, China, 21°34′ N, 111°50′ E, 26 November 2019, immature male, 27.5 mm DML, coll. GBSS. OUC-202103200303, Lingshui, Hainan, China, 18°25′ N, 109°59′ E, 20 March 2021, mature male, 56.2 mm DML, coll. GBSS. OUC-202103200305, Lingshui, Hainan, China, 18°25′ N, 109°59′ E, 20 March 2021, immature female, 43.0 mm DML, coll. GBSS. OUC-202106090301, Zhangzhou, Fujian, China, 23°19′ N, 117°23′ E, 9 June 2021, submature female, 59.5 mm DML, coll. GBSS. OUC-202106090302, Zhangzhou, Fujian, China, 23°19′ N, 117°23′ E, 9 June 2021, submature female, 49.4 mm DML, coll. GBSS. OUC-202106090307, Zhangzhou, Fujian, China, 23°19′ N, 117°23′ E, 9 June 2021, submature female, 55.9 mm DML, coll. GBSS.

*Etymology:* Species epithet *sparsus* is derived from Latin *sparsus*, meaning 'spotty'. The name indicates the presence numerous small black spots on subdermal layer of arms.

*Diagnosis:* Small to medium-sized species (DML 27.5–76.4 mm, TW 5.8–46.3 g). Mantle ovoid. Arms long, approximately three to five times mantle length. Dorsal arms longest and most robust, arm formula mostly 1 > 2 > 3 > 4 or 1 > 2 > 4 > 3. Enlarged suckers inconspicuous in mature males, typically from 8 to 15th on arms I. Webs thin, narrow and easily damaged. Webs of moderate depth, dorsal webs deepest, deepest 9.1 to 16.9% of arm length. Funnel of moderate length, free funnel moderate (36.1–57.6% funnel length). Gills with 10–11 lamellae per demibranch. Mature ovarian eggs small and numerous (> 15,000). Hectocotylized arm 59–80 suckers. Ligula small, 3.1–4.2% arm length. Calamus small, 23.1–29.2% of ligula length. Spermatophores 5–8. Fresh and fixed specimens pale reddish-brown in color. Skin smooth, thin, and easily damaged. Numerous small black spots clearly scattered on subdermal layer of arms. Many small white spots present on dorsal surfaces of mantle and bases of arms in fresh high-quality specimens.

*Description:* The following description is based on six males (five mature and one submature) and nine females (four mature, three submature and two immature). Some characteristics were described on the basis of partial individuals (e.g., total length (TL) and arm length (AL), which were described on the basis of individuals with intact arms). Measurements and counts are summarized in Supplementary Tables S5 and S6.

*External features:* Small to medium-sized species (TL 143.5–293.3–383.5; DML 27.5–55.2–76.4; TWg 5.8–27.2–46.3) (Fig. 3A, B). Mantle elongated, dorsally longer than wide (MWI 53.8–63.5–78.2). Head narrower than mantle (HWI 21.7–29.5–37.2; HMWI 32.6–46.7–59.9). Eyes slightly prominent and large (ELI 7.1–9.0–11.3). Funnel moderate, with moderate-sized free funnel (FLI 22.6–30.2–38.3; FFI 36.1–48.3–57.6) (Fig. 3B). Arms moderate in length and width (approximately 3–5 times mantle length, AWI 10.1–13.7–18.5) and tapering to tips (Fig. 3A). Dorsal arms longest and most robust, arm formula mostly 1 > 2 > 3 > 4 or 1 > 2 > 4 > 3. Webs thin, narrow and moderate in depth (WDI 9.1–14.2–16.9), dorsal webs deepest, ventral webs shallowest. Slightly enlarged suckers typically from 8 to 15th on arm I (enlarged sucker diameter index (LSDI) 6.4–7.4–9.0; NSDI 7.9–9.6–11.1). Diameter of enlarged suckers 1.2–1.4 times that of adjacent normal suckers. Right third arm of mature males hectocotylized, much shorter than opposite arm (OAI 48.9–53.6–57.8). Sucker counts 59–80 on hectocotylus of mature male specimens. Ligula small (LLI 3.1–3.6–4.2), with numerous transverse laminae and a small conspicuous conical calamus (CaLI 23.1–25.6–29.2) (Fig. 3D). Gills with 10–11 lamellae per demibranch.

Fresh and fixed specimens pale reddish-brown in color. Skin smooth, thin, and easily damaged (Fig. 3A, B). Many small white spots present over dorsal surfaces of mantle and bases of arms in fresh specimens of high quality (Fig. 3C). Numerous chromatophores clearly scattered under the skin on dorsal surface of arms (Fig. 3F).

*Reproductive system:* Male reproductive system illustrated in Fig. 3I. Male terminal organ hollow tube, thick, diverticulum muscular, curved with swollen diverticulum. Vas deferens short. Testis moderate in size. Spermatophore storage sac moderate in length, full of mature spermatophores (SpN 5–8). Spermatophores moderate to long (SpLI 32.7–52.3) (Fig. 3J). Female reproductive system illustrated in Fig. 3K. Mature ovary oval. Proximal and distal oviducts long and thin. Mature ovarian eggs numerous and very small (EgLI 1.3–2.2; EgWI 0.2–0.5) (Fig. 3L).

*Digestive system:* Digestive system illustrated in Fig. 3M. Buccal mass large. Anterior salivary glands comprise approximately one-third of buccal masses. Posterior salivary glands large, approximately equal to buccal mass length. Caecum with 1.5 whorl. Intestine moderate with moderate anus. Anal flaps very small (Fig. 3E). Digestive gland well developed. Stomach well developed. Ink sac distinct and narrow, embedded in surface of digestive gland (Fig. 3M).

Chitinous beaks dark brown. Upper beak (Fig. 3N) with a short-hooked rostrum, narrow hood, short wings, and moderate lateral wall. Lower beak with a short rostrum, narrow hood, and long and narrow wings (Fig. 3O, P). Radulae with seven transverse rows of teeth and two rows of marginal plates (Fig. 3Q, R). Three lateral teeth were flanked on either side of one central rachidian tooth, i.e., a 3·1·3 formula. Rachidian tooth multi-cuspid, and with one or two sharp lateral cusps

on each side of median cone. Lateral cusps migrating from lateral to medial position over two rows, every three or four forming a repeating unit. First lateral tooth smallest, with one medial cusp, second lateral tooth with wide heel, one dagger-like cusp, base concave, third lateral tooth with a long blunt cusp, short base.

*Geographical distribution:* This species was found in the coastal waters of Fujian, Guangdong and Hainan. Previous reported specimens with similar characteristics from Nha Trang, South Vietnam (Kaneko et al. 2008) under the name *Callistoctopus* sp. 1 may prove to be conspecific.

*Remarks: Callistoctopus sparsus* sp. nov. was originally reported by Kaneko et al. (2008) from Nha Trang, South Vietnam (*Callistoctopus* sp. 1). The species was described as having numerous chromatophores scattered on the dorsal surface of the arms but not on the webs, a distinctive characteristic also found in *Ca. sparsus* sp. nov. In addition, characteristics, such as 'gill number of 10–11' 'web formula typically A > B > C > D > E' and 'not distinct enlarged suckers', are also consistent with our *Ca. sparsus* sp. nov., strongly suggesting that these two species are conspecific (Supplementary Table S7). In addition, another unknown species, *Ca.* sp. 3, described by Kaneko et al. (2008), is morphologically similar to *Ca.* sp. 1, differing only in the number of gill lamellae (10–11 for *Ca.* sp. 1 and eight for *Ca.* sp. 3), a feature summarized on the basis of only one submature female individual. The molecular species delimitation analysis based on ASAP in this study indicated that *Ca.* sp. 3 may represent a distinct species separate from *Ca.* sp. 1 and *Ca. sparsus* sp. nov. However, this distinction was not corroborated by the results obtained from the ABGD and bPTP analyses. Consequently, further investigation with additional samples is necessary to determine whether *Ca.* sp. 3 should be classified as a new species (or a subspecies), or simply an individual variation. This additional research should aim to gather more morphological and molecular evidence to support the classification of *Ca.* sp. 3.

## *Callistoctopus gracilis* sp. nov. (Fig. 4; Supplementary Tables S8 and S9)

### Type and voucher materials

*Holotype*. OUC-201911260302, Yangjiang, Guangdong, China, 21°34′ N, 111°50′ E, 26 November 2019, mature male, 62.2 mm DML, coll. GBSS.

*Paratypes*. OUC-201911260301, Yangjiang, Guangdong, China, 21°34′ N, 111°50′ E, 26 November 2019, mature female, 84.4 mm DML, coll. GBSS.

*Other materials*. OUC-200802240301, Yangjiang, Guangdong, China, 21°34 N, 111°50′ E, 24 February 2008, mature female, 84.8 mm DML, coll. GBSS.

*Etymology:* Species epithet *gracilis* is derived from Latin *gracilis* meaning 'slender', 'thin', 'simple', 'without ornaments'. The name denotes the smooth body surface and the thin, slender shape of the mantle of this species.

*Diagnosis:* Small to medium-sized species (DML 62.2–84.8 mm, TW 15.1–39.6 g). Mantle very elongated and finger-like. Head narrow. Eyes small. Arms long, dorsal arms longest (typically 1 > 2 > 3 > 4). Arms easily detached. Arm suckers biserial and small. Enlarged suckers absent in mature males. Webs very shallow, depths subequal, deepest web approximately 5.1% of arm length. Funnel moderate and narrow, free funnel very short (27.1–31.3% funnel length). Gills typically with 11 lamellae per demibranch. Branchial heart large and conspicuous. Mature ovarian eggs very large with low numbers (approximately 26). Ligula moderate (approximately 5.5% of arm length), with a moderate calamus (around 40.0% of ligula length). Spermatophores 3 in storage sac of the only mature male. Fixed specimens dark brown. Skin soft and smooth.

*Description:* The following description is based on one mature male and two mature females. Some characteristics were described on the basis of partial individuals (e.g., total length (TL) and arm length (AL), which were described on the basis of individuals with intact arms). Measurements and counts are summarized in Supplementary Tables S8 and S9.

*External features:* Small to medium-sized species (TL 314.1–379.0–445.7; DML 62.2–77.1–84.8; TWg 15.1–24.5–39.6) (Fig. 4A, B). Mantle elongated, finger-like, dorsally much longer than wide (MWI 40.2–44.6–46.8). Pallial aperture narrow, approximately 1/2 mantle length (Fig. 4C). Head narrower than mantle (HWI 18.6–22.1–28.1; HMWI 39.9–49.6–60.1). Eyes prominent and small (ELI 3.1–3.3–3.7) (Fig. 4A). Funnel moderate and narrow, with very short free funnels (FLI 34.1–35.7–38.6; FFI 27.1–28.7–31.3) (Fig. 4C). Arms long (arm mantle index (AMI1) 418.5 in male specimen), very slender (AWI 6.8–7.4–7.7) and tapering to tips. Dorsal arm longest, arm formula 1 > 2 > 3 > 4. Webs very shallow (WDI 5.1), depths subequal, webs between ventral arms shallowest (Fig. 4A, B). Arms easily detached. Arm autotomy probable. Arm suckers small, biserial and thick-rimmed (NSDI 3.0–3.4–4.2). Enlarged suckers absent in mature males. Right third arm of mature males hectocotylized, much shorter than opposite arm. Sucker counts 49 on hectocotylus of mature male specimen. Ligula moderate (LLI 5.5 in male specimen), with a conspicuous, conical calamus (CaLI 40.0 in male specimen). Spermatophore groove distinct (Fig. 4D). Gills with 11 lamellae per demibranch. Branchial heart large and conspicuous (Fig. 4E). Fresh and fixed specimens dark or light brown in color. Skin smooth. Patch and groove skin sculptures absent (Fig. 4A, B).

*Reproductive system:* Male reproductive system illustrated in Fig. 4I. Male terminal organ hollow tube, thick,

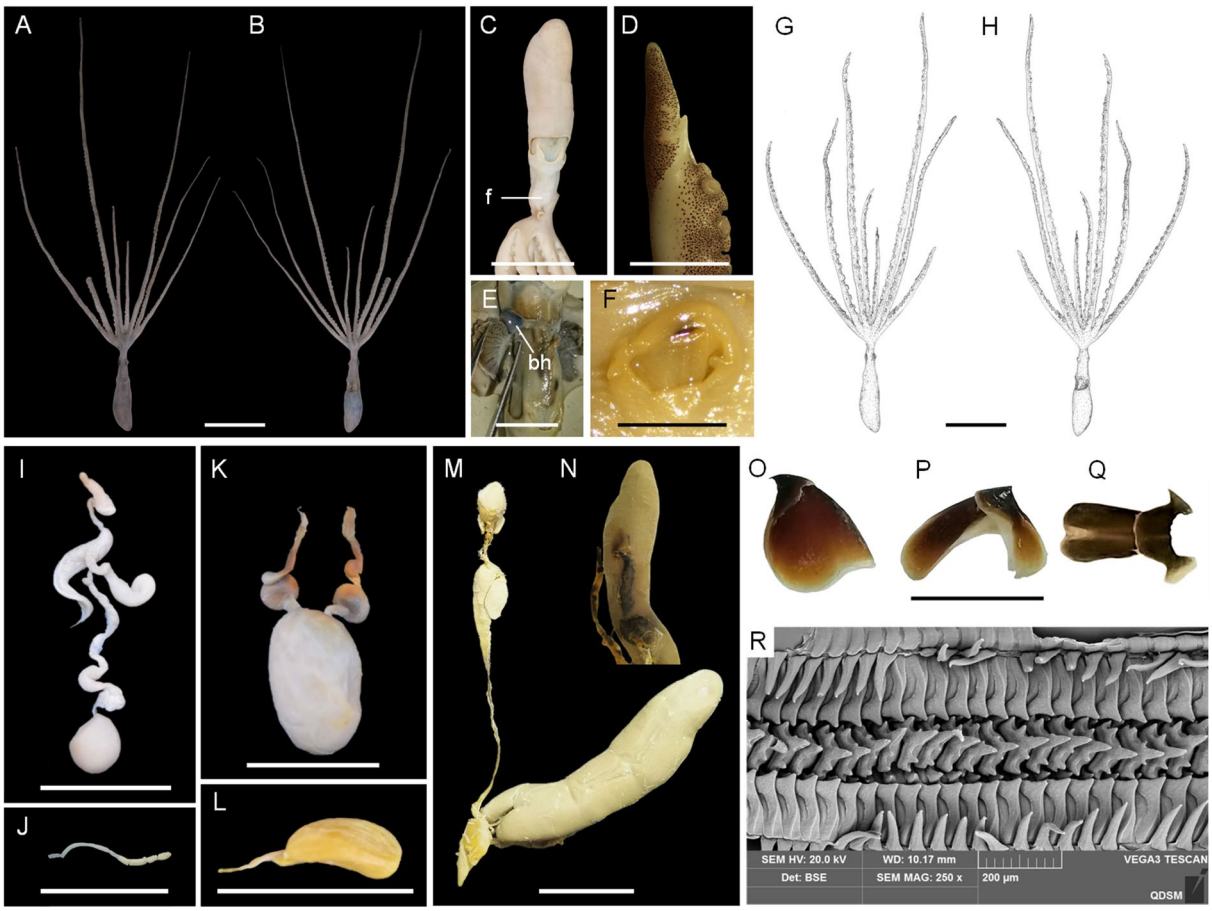


**Fig. 4** *Callistoctopus gracilis* sp. nov. from alcohol-fixed specimens. **A** Dorsal view (OUC-201911260301). **A** Ventral view (OUC-201911260301). **C** Dorsal view of partial body (OUC-201911260301). **D** Hectocotylus of mature male (OUC-201911260302). **E** Anatomical structure (OUC-201911260301). **F** Anus (OUC-201911260302). **G**, **H** Line drawings of dorsal (**G**) and ventral (**H**) views of fixed specimens. **I** Male reproductive system (OUC-201911260302). **J** Spermatophore (OUC-201911260302). **K** Female reproductive system (OUC-201911260301). **L** Single laid egg (OUC-201911260301). **M** Whole view of digestive system (OUC-201911260301). **N** Ink sac (OUC-201911260301). **O**–**Q** Lateral (**O**) view of upper beak (OUC-201911260301), lateral (**P**) and top (**Q**) views of lower beak (OUC-201911260301). **R** Radula of male specimen (OUC-201911260302). *bh* branchial heart, *f* funnel

diverticulum muscular (TOLI 14.3 in male specimen), curved with swollen diverticulum. Vas deferens slender and coiled. Testis moderate in size. Spermatophore storage sac moderate in length. Spermatophores moderate in length (SpLI 28.0–34.6) (Fig. 4J). Only three spermatophores (SpN 3) in storage sac of the only mature male.

Female reproductive system illustrated in Fig. 4K. Mature ovary elongated and occupying the posterior nearly half of the mantle cavity. Distal oviducts moderate, thick, and muscular. Mature eggs huge with short stalks, capsule length around 10.0–16.0 mm (EgLI 11.8–18.9, EgWI 4.5–7.8) (Fig. 4L). Approximately 26 mature ovarian eggs present in mature female examined.

*Digestive system:* Digestive system illustrated in Fig. 4M. Buccal mass large. Anterior salivary glands approximately one-third of buccal mass. Posterior salivary glands large, approximately equal to buccal mass length. Caecum with one whorl. Intestine long with small anus (Fig. 4M). Anal flaps very small (Fig. 4F). Digestive gland elongated and well developed. Ink sac distinct and swollen, embedded in surface of digestive gland (Fig. 4N).

Chitinous beaks dark brown. Upper beak (Fig. 4O) with a short-hooked rostrum, narrow hood, short wings, and broad lateral wall. Lower beak with a short rostrum, narrow hood, short and moderately broad wings with slightly curved lateral walls (Fig. 4P, Q). Radulae with seven transverse rows of teeth and two rows of marginal plates (Fig. 4R). Three lateral teeth flanked on either side of one central rachidian tooth, i.e., a 3·1·3 formula. Rachidian tooth multi-cuspid, and with one or two sharp lateral cusps on each side of median cone. Lateral cusps migrating from lateral to medial position over two rows, every three or four forming a repeating unit. First lateral tooth smallest, with one medial cusp, second lateral tooth with wide heel, one dagger-like cusp,

base concave, third lateral tooth with a long blunt cusp, short base.

*Geographical distribution:* The specimens were collected in the South China Sea near Guangdong in this study, and also reported from Hong Kong and Nha Trang, Vietnam (Kaneko et al. 2008; Norman and Hochberg 1994; Voss and Williamson 1971). This narrow distribution is probably because of the egg size that produce benthic larvae with a limited dispersal range.

*Remarks: Callistoctopus gracilis* sp. nov. shares many morphological similarities with *Octopus* sp. B reported by Voss and Williamson (1971), *O. fusiformis* reported by Dong (1987), *Octopus* sp. 1 reported by Norman and Hochberg (1994), and *Callistoctopus* sp. 2 reported by Kaneko et al. (2008). These earlier studies highlighted distinctive characteristics of the species, such as an elongated body and arms, spindle-shaped mantle, narrow head, very short free funnel and specific quantitative traits like 10–11 lamellae per outer demibranch and 50–60 suckers on the hectocotylized arm. Based on these similarities, it is suggested that these species may be conspecific (See "Discussion" section and Supplementary Table S10).

It is important to highlight that this species has previously been mistaken for *O. minor*, as reported by Kaneko et al. (2008). Despite both species having elongated bodies with smooth, unmarked surfaces and coexisting in the same geographical area (*O. minor* is found throughout coastal Asia), they may be differentiated morphologically. Specifically, *Ca. gracilis* sp. nov. may be distinguished from *O. minor* by its more elongated mantle, head, and arms, smaller free funnel, smaller ligula and lack of transverse laminae.

## Species delimitation

In this study, three delimitation analyses were fairly consistent, and the analysis results using ABGD and bPTP were identical, differing slightly from ASAP in terms of the number of delineated molecular operational taxonomic units (MOTUs) (10 MOTUs in ABGD and bPTP, 11 MOTUs in ASAP). Overall, all delimitation methods confirmed the recognition of three separate MOTUs for the target species. In the ABGD and bPTP analyses, two unknown *Callistoctopus* species (*Ca.* sp. 2 and *Ca.* sp. 3) were identified as *Ca. sparsus* sp. nov. In contrast, they were assigned to two different MOTUs whereas in the ASAP analysis. *Callistoctopus* sp. 1 was identified as *Ca. gracilis* sp. nov.

## Molecular data and phylogenetic analyses

A comparison of all COI gene sequences of *Callistoctopus* species (Fig. 5A) showed that sequence identities ranged from 82.46% to 98.88%, with 7 (between *Ca. sparsus* sp. nov. and *Ca.* sp. 2) to 110 (between *Ca. xiaohongxu* and *Ca. luteus*) unmatched nucleotide positions. *Callistoctopus gracilis* sp. nov. was the most similar to *Ca.* sp. 1 AB385875, with 16 unmatched nucleotides (97.45% sequence identity). It differed from other *Callistoctopus* species and *O. minor* sequences by 59–93 nucleotides (80.17–90.59% sequence identities). *Callistoctopus sparsus* sp. nov. was similar to *Ca.* sp. 2 AB385875 and *Ca.* sp. 3 AB385877, with 7 and 30 unmatched nucleotides (98.88% and 95.22% sequence identity, respectively) (Fig. 5B). It differed from other *Callistoctopus* species and *O. minor* sequences by 63–100 nucleotides (84.05–89.95% sequence identity). The intraspecific sequence identities ranged from 99.68% to 100%, with 0–2 unmatched nucleotides for the three new species. In addition, although *O. minor* is not currently classified in the genus *Callistoctopus*, it was close to other *Callistoctopus* species in terms of nucleotide mismatch and similarity, with a range of variation between 61–95 and 84.85–90.27%, respectively. However, the range of interspecific variation among *Callistoctopus* species was between 7–110 and 82.46–98.88%, respectively (Fig. 5B).

As showed in Supplementary Table S11, the Kimura 2-parameter distances of the COI sequence within species ranged from 0 to 0.0129, with an average distance of 0.0048; the highest distance of 0.0129 was found in *O. minor*. The interspecific genetic distance ranged from 0.0447 to 0.1552, with an average of 0.1209, which was 25 times the mean intraspecific genetic distance. The largest interspecies distance of 0.1552 was found between *Ca. luteus* and *Ca. macropus* and the lowest was found between *Ca. ornatus* and *Ca. macropus*.

In our analyses, all our target species (*Ca. paucilamellus* sp. nov., *Ca. sparsus* sp. nov. and *Ca. gracilis* sp. nov.) appeared as three separate clusters with strong support values, and were all located in the genus *Callistoctopus*. Two phylogenetic trees constructed using maximum likelihood (ML) and Bayesian inference (BI) methods based on the four genes had similar topologies, except for the position of *Robsonella fontaniana* and *Cistopus taiwanicus* (Fig. 6). Three *Callistoctopus* species (AB385875, AB385876, and AB385877) were not initially identified by Kaneko et al. (2008) at the species level, and were grouped into two of our clusters with high support values. All our new species grouped into two distinct clades with strong support values. In the first clade, *Ca. gracilis* sp. nov. formed a sister relationship with *Ca.* sp. 1 (AB385875). Together with *Ca. xiaohongxu* and *Ca. tenuipes*, these four species constituted a sister group to *Ca. paucilamellus* sp. nov. (bootstrap support [BS] = 100, posterior probability [PP] = 1). This clade exhibited a close relationship with *O. minor* and *Ca. aspilosomatis*. The second clade showed that *Ca. sparsus* sp. nov. was closely associated with *Ca.* sp. 2 (AB385876) and *Ca.* sp. 3 (AB385877) (bootstrap [BS] = 100, Posterior Probability

B

| | Length | GC content | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 *C. gracilis* sp. nov. | 627 bp | 35.09% | - | 90.27 | 88.36 | 87.24 | 89.31 | 90.59 | 85.49 | 85.17 | 86.92 | 88.84 | 97.45 | 87.56 | 87.08 |
| 2 *C. paucilamellus* sp. nov. | 627 bp | 34.45% | 61 | - | 89.31 | 87.72 | 89.95 | 90.27 | 85.49 | 84.37 | 87.08 | 90.27 | 88.68 | 88.36 | 88.20 |
| 3 *C. sparsus* sp. nov. | 627 bp | 35.73% | 73 | 67 | - | 89.95 | 89.63 | 89.79 | 84.05 | 84.69 | 88.04 | 88.04 | 88.36 | 98.88 | 95.22 |
| 4 *C. aspilosomatis* | 627 bp | 34.93% | 80 | 77 | 63 | - | 88.36 | 90.11 | 82.78 | 82.78 | 87.24 | 88.20 | 86.76 | 89.31 | 88.84 |
| 5 *C. macropus* | 627 bp | 34.61% | 67 | 63 | 65 | 73 | - | 95.69 | 83.57 | 84.53 | 86.12 | 89.00 | 87.88 | 88.68 | 87.56 |
| 6 *C. ornatus* | 627 bp | 33.97% | 59 | 61 | 64 | 62 | 27 | - | 84.69 | 84.69 | 86.44 | 89.63 | 89.15 | 88.84 | 88.84 |
| 7 *C. tenuipes* | 619 bp | 34.58% | 91 | 91 | 100 | 108 | 103 | 96 | - | 84.44 | 83.09 | 85.33 | 84.69 | 83.57 | 84.37 |
| 8 *C. xiaohongxu* | 595 bp | 33.56% | 93 | 98 | 96 | 108 | 97 | 96 | 96 | - | 82.46 | 84.85 | 84.37 | 84.05 | 84.53 |
| 9 *C. luteus* | 627 bp | 36.20% | 82 | 81 | 75 | 80 | 87 | 85 | 106 | 110 | - | 87.72 | 89.00 | 88.84 | 88.84 |
| 10 *O. minor* | 627 bp | 33.33% | 70 | 61 | 75 | 74 | 69 | 65 | 92 | 95 | 77 | - | 87.72 | 86.92 | 87.40 |
| 11 *C. sp.1* | 627 bp | 35.73% | 16 | 71 | 73 | 83 | 76 | 68 | 96 | 98 | 69 | 77 | - | 89.15 | 89.15 |
| 12 *C. sp.2* | 627 bp | 35.89% | 78 | 73 | 7 | 67 | 71 | 70 | 103 | 100 | 70 | 82 | 68 | - | 96.33 |
| 13 *C. sp.3* | 627 bp | 34.93% | 81 | 74 | 30 | 70 | 78 | 70 | 98 | 97 | 70 | 79 | 68 | 23 | - |

*Intraspecifc sequence identities (unmatched nucleotides): *C. gracilis* 99.83-100% (0-1), *C. paucilamella* 100% (0), *C. sparsus* 99.68-100% (0-2)

**Fig. 5** **A** Comparison of COI gene sequences of three new species with their most closely related congeners. Numbers represent unmatched nucleotide positions in the alignment. Dots (.) and dashes (−) represent matched sites and deletions, respectively. **B** Pairwise comparison of COI gene sequences of 12 *Callistoctopus* species and *Octopus minor*. Above and below the diagonal indicate sequence identities and numbers of unmatched nucleotides, respectively

[PP] = 1). Additionally, *Ca. luteus* and *Ca. macropus* were positioned at the base of the *Callistoctopus* group.

Our analyses consistently revealed a clade comprising *Callistoctopus*, *Grimpella thaumastocheir*, *Macroctopus maorum*, and *Pinnoctopus cordiformis*. This group is closely related to a clade that includes *Pteroctopus tetracirrhus*, *R. fontaniana*, and *Scaeurgus unicirrhus*, although there are minor differences in the topology within these three species. Additionally, the phylogenetic position of *Ci. taiwanicus* was unresolved in the two topologies resulting from BI and ML analyses. In this study, the Amphitretidae and the genus *Octopus* were found to be polyphyletic taxa. Instead, Megaleledonidae and Enteroctopodidae each formed a well-supported monophyletic group in both analyses.

## Discussion

*Callistoctopus* is characterized by relatively high species diversity, comprising a total of 13 documented species. *Callistoctopus* was originally described as a large, robust and muscular animal (Taki 1964), and later expanded to 13 species (Gould 1852; Li et al. 2022; Norman 1993; Norman and Sweeney 1997; Risso 1826; Sasaki 1929; Stranks 1990; Voss 1979; Zheng et al. 2022). With the addition of the three new species in the present study, the genus was expanded to 16 species (Fig. 1).

Among these 16 species, *Ca. lechenaultii* needs to be fully described. The remaining species may be roughly divided into two groups according to web depth (Norman 1993). The first group included *Ca. graptus*, *Ca. xiaohongxu*, *Ca. luteus*, *Ca. rapanui*, *Ca. dierythraeus* and *Ca. alpheus*, whose deepest webs always accounted for approximately 15% or more, typically 20%, of the longest arm length. The remaining species formed the second group, each with a shallow web (the deepest web was always less than 15%, typically 10%, of the longest arm length). The three new species identified in this study belong to the latter grouping (Supplementary Tables S4, S6, S9).

In addition to variations in web depth among species of *Callistoctopus*, distinguishing characteristics, such as gill counts, hectocotylized arm length, sucker count on the hectocotylized arm, egg size and count, and spermatophore size and count, are crucial for species differentiation within

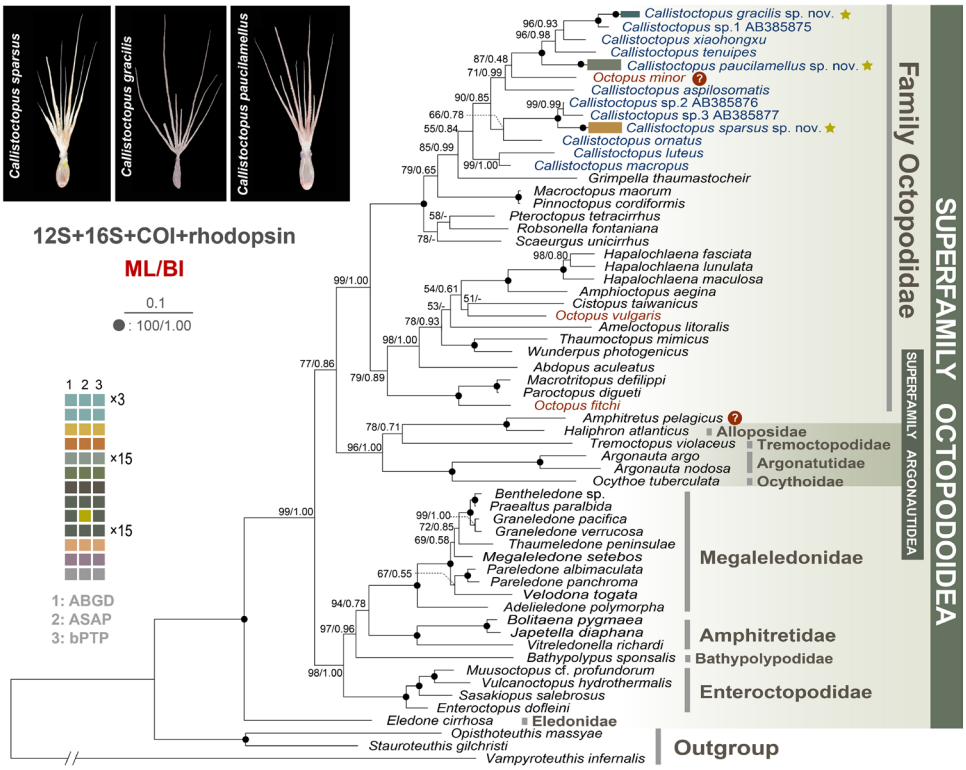


**Fig. 6** Phylogenetic tree based on partial 12S rRNA, 16S rRNA, COI and Rhodopsin gene sequences, showing the systematic positions of *Ca. paucilamellus* sp. nov., *Ca. sparsus* sp. nov., and *Ca. gracilis* sp. nov. The numbers separated by "/" near the nodes represent bootstrap values of maximum likelihood (ML) and posterior probabilities of Bayesian inference (BI) analyses, respectively. Fully supported (100%/1.00) nodes are marked with solid circles. "–" indicate mismatches in topologies between ML and BI analyses. Question marks marked in red indicate species whose taxonomic status is considered questionable in this study. Colored squares in the bottom left are the results of *Callistoctopus* species delimitation analyses (1: ABGD, 2: ASAP, 3: bPTP), with each color representing a distinct candidate species according to the respective method

the genus. In comparison, consistent characteristics across the entire genus include well-developed ligula and calamus in mature males, inconspicuous enlarged suckers, distinct anal flaps and ink sac, a smooth body surface, and bright coloration or spotting patterns (Norman et al. 2013). These characteristics collectively serve as reliable identifiers for the genus *Callistoctopus*.

## Morphological analysis

### Comparison of *Callistoctopus paucilamellus* sp. nov. with similar congeners

*Callistoctopus paucilamellus* sp. nov. is characterized by a low number of gill lamellae per demibranch (7–8) (Fig. 7A). This feature clearly distinguishes it from most congeners (9–14). Additionally, based on its body size (approximately 30–60 mm in mantle length, 200–300 mm in total length), body color (red to reddish-brown), skin (smooth), eggs (large-size and numerous) and fewer gill lamellae per demibranch (7–8), there are two species that should be compared with *Ca. paucilamellus* sp. nov., namely *Ca. xiaohongxu* (Zheng et al. 2022) and *Ca. tenuipes* (Li et al. 2022) (Supplementary Table S12).

*Callistoctopus xiaohongxu* may be clearly separated from *Ca. paucilamellus* sp. nov. by its deeper web (WDI 15.7–22.9 vs. 10.5–14.9), longer funnel (FLI 51.0–68.5 vs. 28.0–43.9), longer hectocotylized arm (HAMI 155.5–184.2 vs. 106.7–127.0), more suckers in hectocotylized arm (hectocotylized arm sucker count (HASC) 70–83 vs. 63–65), larger ligula (LLI 7.0–11.6 vs. 3.3–5.5), smaller calamus (CaLI 26.3–31.6 vs. 36.4–37.5) and longer spermatophore (SpLI 76.4–125.2 vs. 35.9–38.1) (Zheng et al. 2022).

*Callistoctopus tenuipes* may be distinguished from *Ca. paucilamellus* sp. nov. by its longer hectocotylized arm (HAMI 148.9–193.8 vs. 106.7–127.0), larger and more eggs (EgLI 25.0–26.4, EgWI 7.7–7.9, egg number (EgN) ca. 110 vs. EgLI 12.1–20.4, EgWI 3.5–6.5, EgN 49), and longer spermatophore (SpLI ca. 144.7 vs. 35.9–38.1) (Li et al. 2022).

### Comparison of *Callistoctopus sparsus* sp. nov. with similar congeners

This species is an elongated species with a shallow web and small eggs, characteristics shared by many species of the genus *Callistoctopus* (i.e., *Ca. ornatus*, *Ca. nocturnus*, *Ca. aspilosomatis*, and *Ca. macropus*). However, *Ca. sparsus* sp. nov. may be distinguished from these species by its smaller ligula (LLI 3.1–3.6–4.2 vs. 4.3–8.8) and numerous small black spots on the subdermal layer of the arms. With the exception of *Ca. sparsus* sp. nov., two other species, *Ca. xiaohongxu* and *Ca. tenuipes,* exhibit

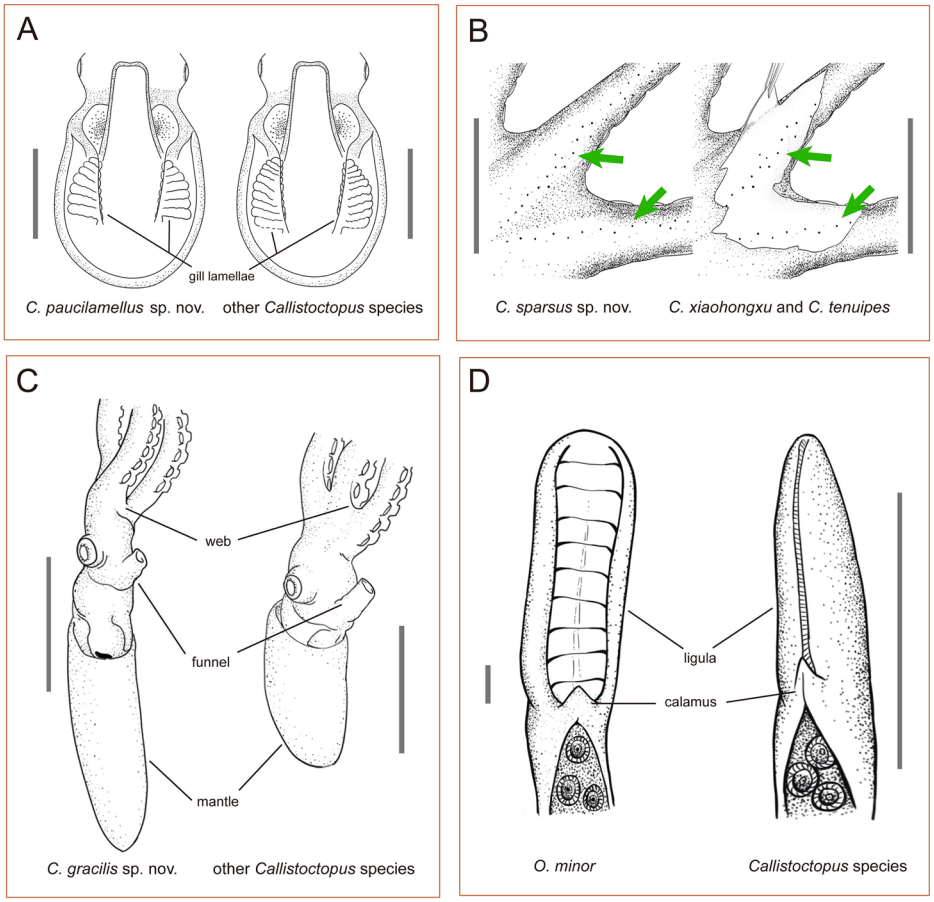


**Fig. 7** Schematic comparison of the four structures of three new species, *Octopus minor*, and related species. **A** Coronal plane of mantle. **B** Dorsal arms. Red arrows indicate chromophores located in the subdermal layer of the arms, which can be observed through the epidermis (left) and only when the epidermis is removed (right). **C** Lateral view of mantle, head and the base of arms. **D** Hectocotylized arm. Scale bars: **A**, **B** = 20 mm, **C** = 40 mm, **D** = 2 mm

scattered chromatophores on their arms, and are compared with *Ca. sparsus* sp. nov.

*Callistoctopus xiaohongxu* may also be distinguished from *Ca. sparsus* sp. nov. by its lower gill lamellae per demibranch (gill counts (GC) 8–9 vs. 10–11), longer funnel (FLI 51.0–68.5 vs. 22.6–38.3), longer ligula (LLI 7.0–11.6 vs. 3.1–4.2), much larger and fewer eggs (EgLI 17.4–27.1, EgWI 4.0–8.3, EgN 65 vs. EgLI 1.3–2.2, EgWI 0.2–0.5, EgN ca. 15,000), and longer spermatophore (SpLI 76.4–125.2 vs. 32.7–52.3).

*Callistoctopus tenuipes* possesses larger and fewer eggs (EgLI 25.0–26.4, EgWI 7.7–7.9, EgN ca. 110 vs. EgLI 1.3–2.2, EgWI 0.2–0.5, EgN ca. 15,000), as well as longer spermatophores (SpLI 144.7 vs. 32.7–52.3) than *Ca. sparsus* sp. nov.

It is noteworthy that the epidermis of *Ca. sparsus* sp. nov. was thin enough to allow clear visualization of chromatophores located in the subdermal layer of the arms. In contrast, the epidermis of *Ca. xiaohongxu* and *Ca. tenuipes* was thicker, making the chromatophores unobservable through visual inspection alone (Fig. 7B). The separation of these two species from *Ca. sparsus* sp. nov. was further supported by the molecular data (Fig. 5).

### Comparison of *Callistoctopus gracilis* sp. nov. with similar congeners

This species exhibits a high level of morphological resemblance to several previously uncertain species documented in earlier studies (*Octopus* sp. B in Voss and Williamson 1971, *Octopus* sp. 1 in Norman and Hochberg 1994 and *Callistoctopus* sp. 2 in Kaneko et al. 2008). We summarized the main morphological features mentioned, noting that due to variations in the extent of detail in the descriptions, some morphological traits of the uncertain species may not have been specified. Nevertheless, these uncertain species collectively display notably short free funnels, an arm formula of 1 > 2 > 3 > 4, 11–12 gill lamellae on each demibranch, a hectocotylized arm considerably shorter than the opposing arm with 49–61 sucker counts, and a markedly narrow and elongated mantle, all of which are entirely identical with *Ca. gracilis* sp. nov. in this study (Supplementary Table S10). Furthermore, Dong (19,870 included *O. fusiformis* in his review of the cephalopod fauna of China. We rechecked the records and specimens, and considered them identical to *Ca. gracilis* sp. nov. in the present study (Supplementary Table S10). Despite limited molecular evidence, we suggest

that the aforementioned species should be classified as conspecific with *Ca. gracilis* sp. nov. due to shared morphological characteristics.

In addition, based on its body size (approximately 60–80 mm in mantle length, 300–400 mm in total length), long arm (four times mantle length), gill lamellae per demibranch (11) and large eggs (approximately 10–16 mm), there are two species that should be compared with *Ca. gracilis* sp. nov., namely *Ca. alpheus* Norman 1993 and *Ca. bunurong* Stranks 1990 (Supplementary Table S12).

When compared with *Ca. alpheus*, *Ca. gracilis* sp. nov. has a much shallower web (WDI 5.1 vs. 16.3–21.8–25.0), shorter free funnel (FFI 27.1–28.7–31.3 vs. 30.1–46.7–74.2), fewer suckers in hectocotylized arm (HASC 49 vs. 82–91–97) and shorter spermatophores (SpLI 28.0–34.6 vs. 92.3–93.7–95.1).

*Callistoctopus gracilis* sp. nov. differs mainly from *Ca. bunurong* by having a shallower web (WDI 5.1 vs. 9.4–12.4–14.8), shorter free funnel (FFI 27.1–28.7–31.3 vs. 33.3–58.4), fewer suckers in hectocotylized arm (HASC 49 vs. 70–96), shorter ligula (LLI 5.5 vs. 9.0–9.8–11.8), much longer calamus (CaLI 40.0 vs. 12.8–17.5–22.1) and shorter spermatophores (SpLI 28.0–34.6 vs. 41.2–65.1–102.9).

Collectively, this species is distinct from all other *Callistoctopus* species in its slender body structure, including a slender mantle, arm, head and digestive gland. Also, it possesses the shallowest web (WDI 5.1) and the smallest free funnel (FFI 27.1–31.3) in this genus (Fig. 7C).

#### Comparison of *Callistoctopus* species with *O. minor*

*Octopus minor* may be easily identified by its distinctive hectocotylized arm. The calamus was small but developed. The ligula was well developed, large, and spoon-like with a transverse deep hollow groove. The ligula was typically wider than the narrowest part of the hectocotylized arm. These features differentiated it from *Callistoctopus* species, which have a developed, moderately sized, conical calamus and a moderate to large cylindrical ligula with a shallow to deep groove (Fig. 7D). However, it is important to note that the spoon-like ligula is only found in *O. minor,* and is not present in either the *Callistoctopus* or *Octopus* genera. Therefore, this feature alone cannot be used as a basis for determining the genus to which a species belongs.

Regardless of the unique spoon-like ligula, *O. minor* shared many similarities with species in the genus *Callistoctopus*, including identical elongated body shape, arm formula (1 > 2 > 3 > 4), web formula (A > B > C > D > E), and body color (reddish brown with spots). In particular, *Ca. gracilis* sp. nov. (Kaneko et al. 2008; Sasaki 1929), *Ca. xiaohongxu* (Zheng et al. 2022) and *Ca. tenuipes* (Li et al. 2022) have all been confused with *O. minor,* and have even been mistaken as the juveniles of *O. minor* and sold in fish markets, providing morphological evidence for the close affinity of *O. minor* with *Callistoctopus* species. These close affinities were further supported by the molecular data in our study (Fig. 5).

### Species delimitation

Three uncertain species of *Callistoctopus* (*Ca.* sp. 1–3) identified by Kaneko et al. (2008) were found to be closely related to our new species (Kaneko et al. 2008). The methods used for species delimitation are generally consistent in assigning taxa to MOTUs. However, individual analyses showed deviations (Fig. 6). In the ASAP analysis, *Ca.* sp. 2 and *Ca.* sp. 3 were divided into two different MOTUs. Our morphological analysis supported that *Ca.* sp. 1 of Kaneko et al. (2011) is the same as *Ca. sparsus* sp. nov., and sp. 2 is the identical with *Ca. gracilis* sp. nov. However, our molecular data, species delimitation and subsequent phylogenetic results indicated that *Ca.* sp. 1 of Kaneko et al. (2011) and sp. 2 are conspecific with *Ca. gracilis* sp. nov. and *Ca. sparsus* sp. nov., respectively. *Callistoctopus* sp. 2 and *Ca.* sp. 3 exhibited significant sequence similarity when compared to *Ca.* sp. 1. We analyzed the COIII gene sequence from the same individual as documented in a previous study by Kaneko et al. (2011), which aligns with the molecular results for COI. The conflict between morphological and molecular results is difficult to explain, and one possible reason is that the two species markings may have been confused at some point in the study. The methods used for species delimitation are generally consistent in assigning taxa to MOTUs. However, individual analyses showed deviations (Fig. 6). In the ASAP analysis, *Ca.* sp. 2 and *Ca.* sp. 3 were divided into two different MOTUs, which is supported by morphological studies under the assumption that sp. 1 and sp. 2 were mislabeled, that is, they are morphologically very similar but differ in gill counts.

### Phylogenetic analyses

#### Phylogenetic relationships within the *Callistoctopus* genus

This study presents a relatively extensive phylogenetic examination of *Callistoctopus* species. In addition to the inclusion of three newly discovered species, we investigated 6 of the 13 congeners. In the present study, three new species from the coastal waters of China were recognized, forming strongly supported separate clades and assigned to *Callistoctopus*, which supports the high biodiversity within this genus (Fig. 6). Our analyses showed a close relationship between *Ca. tenuipes* and *Ca. xiaohongxu*, consistent with recent studies by Li et al. (2022) and Zheng et al. (2022). Interestingly, in our study, *Ca. luteus* and *Ca. macropus* were found to be located at the basal position among all the

*Callistoctopus* species analyzed. This finding was not supported by other recent studies (Li et al. 2022; Zheng et al. 2022), and is likely attributed to the use of different molecular markers.

#### Phylogenetic status of *Octopus minor*

As early as 1920, *O. minor* (Sasaki 1920) was first reported as a variant of *O. macropus* Risso 1826 and named *Polypus macropus* var. *minor*, which was subsequently renamed *Polypus variabilis* and divided into three variation types (*Polypus variabilis typicus*, *Polypus variabilis pardalis* and *Polypus variabilis minor*). These three types are not currently accepted, despite evidence indicating a subtle variation in the morphology of *O. minor* among collection sites (Acosta-Jofré et al. 2012; Kaneko et al. 2008, 2011).

To date, fewer studies have been conducted on the phylogenetic status of *O. minor*, and the species is still considered an "unplaced" species, currently provisionally classified within the genus *Octopus* (Norman et al. 2013). However, recent phylogenetic studies, including our own, have revealed that *O. minor* is more closely related to *Callistoctopus* (Ibáñez et al. 2020, 2021; Kaneko et al. 2011; Li et al. 2022; Taite et al. 2023; Zheng et al. 2022). Moreover, some researchers have placed this species directly into *Callistoctopus* (Ibáñez et al. 2020, 2021; Kaneko et al. 2011) (Fig. 6). Specifically, this species was nested within *Callistoctopus* and formed a distinct clade with *Ca. gracilis* sp. nov., *Ca.* sp. 1, *Ca. xiaohongxu*, *Ca. tenuipes* and *Ca. paucilamellus* sp. nov., which is consistent with the findings of Li et al. (2022). Also, the morphological evidence and molecular data in our study support this conclusion.

This observation suggests that *O. minor* is likely not a member of the *Octopus* group, but rather a species belonging to the *Callistoctopus*. Meanwhile, further molecular and morphological studies are needed to comprehensively describe the characteristics of this misidentified species within the catch-all group of *Octopus*.

#### Phylogenetic relationships within the *Octopodidae*

The Octopodidae family currently includes 23 genera and approximately 150 species (World Register of Marine Species, WORMS). We conducted BI and ML analyses, focusing on 17 genera (Fig. 6). In our study, *Callistoctopus* clustered together with *G. thaumastocheir*, than *M. maorum* and *Pi. cordiformis*, and shared a close relationship with *Pt. tetracirrhus*, *R. fontaniana* and *S. unicirrhus* (Fig. 6). In recent decades, the relationship between *M. maorum* and *Pi. cordiformis* has always been a subject of controversy. O'Shea (1999) proposed that *Pi. cordiformis* (Quoy and Gaimard 1832) should be considered as the senior synonym of *M. maorum* Hutton, 1880, and established a neotype (NIWA 43044, H-668) due to the lack of original type material. However, Norman and Hochberg (2005) argued that the original description of *Pi. cordiformis* by Quoy and Gaimard in 1832 actually corresponds more closely to *Enteroctopus zealandicus* based on morphological evidence. Norman et al. (2013) further emphasized the unresolved status of *Pi. cordiformis*, suggesting that it was a misidentification of *M. maorum* rather than a senior synonym. A recent study by Ibáñez et al. (2020) investigated the taxonomy and phylogenetic relationships of octopuses in New Zealand using eighty-eight specimens, and reevaluated the neotype specimen of *Pi. cordiformis* (NIWA 43044, H-668). This study demonstrated that *Pi. cordiformis* and *E. zealandicus* are distinct species, and proposed that *Pi. cordiformis* and *Pi. kermadecensis* should be retained as the correct names. Our study supported a close relationship between *Pi. cordiformis* and *M. maorum* with strong support value.

In addition, the taxonomic relationship between *Pinnoctopus* and *Callistoctopus* has been a topic of debate. O'Shea (1999) proposed that both *Macroctopus* and *Callistoctopus* should be considered junior synonyms of *Pinnoctopus*. Subsequent studies by Norman and Hochberg (2005) and Reid and Wilson (2015) supported the placement of *Pi. kermadecensis* within the genus *Callistoctopus*. More recently, there have been studies indicating a close relationship between *Grimpella*, *Pinnoctopus* and *Callistoctopus* (Ibáñez et al. 2020). In a study by Rosa et al. (2024), *Callistoctopus* species were directly referred to as *Pinnoctopus*. However, our study did not provide direct evidence of the closest affinity between *Pinnoctopus* and *Callistoctopus*, suggesting that further investigation is needed. More informative molecular data, such as at the transcriptome or genome level, may help clarify the relationship between these two genera.

Our findings recovered a clade containing *Callistoctopus*, *Grimpella*, *Macroctopus*, *Pinnoctopus* and *Scaeurgus* consistent with prior research (Ibáñez et al. 2020; Sanchez et al. 2018; Strugnell et al. 2014). However, the specific relationships among these species remain unresolved. Our phylogenetic analyses using ML and BI methods yielded different results. The former supported the topology of ((*Pt. tetracirrhus* + *R. fontaniana*) + *S. unicirrhus*), whereas the latter favored ((*Pt. tetracirrhus* + *S. unicirrhus*) + *R. fontaniana*). This clade was then positioned at the base of the (*Callistoctopus*/*Grimpella*/*Macroctopus*/*Pinnoctopus*/*Scaeurgus*) group. Notably, Strugnell et al. (2014) constructed phylogenic analyses using three gene datasets, resulting in three distinct topologies. Subsequent studies by Sanchez et al. (2018) and Ibáñez et al. (2020) reported varying outcomes. Considering the limited species within these genera, augmenting the dataset with a broader range of genes to improve resolution may be an effective way to elucidate their interrelationships.

Furthermore, this study provided strong support for the monophyly of certain higher taxa, including Megaleledonidae and Enteroctopodidae. Nonetheless, there are some taxa, such as *Cistopus*, *Amphitretus*, and even the superfamily Argonautoidea, that exhibited an uncertain phylogenetic position, contradicting earlier studies (Ibáñez et al. 2020; Sanchez et al. 2018; Strugnell et al. 2014). These inconsistencies may be due to the lack of molecular evidence for most species in these genera, and the limited informativeness of limited nuclear and mitochondrial genes in resolving higher phylogenetic relationships (Taite et al. 2023). Therefore, further work is needed, including more molecular sequences, additional taxon sampling, more informative mitochondrial and nuclear data, and a thorough evaluation of morphological characteristics, to resolve these inconsistencies and improve our understanding of octopod phylogeny.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s42995-024-00258-6.

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**Author contributions** YT conceived the idea, designed the study, analyzed the data, and wrote the manuscript. XZ and CL supervised the studies, collaborated in writing and provided editorial advice. All authors have read and commented on the manuscript.

**Data availability** The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/genbank/ (OP104744–OP104776, PP987172–PP987175).

## Declarations

**Conflict of interest** The authors declare that they have no conflict of interest.

**Animal and human rights statement** The authors declare that all applicable international, national, and or institutional guidelines for sampling, care, and experimental use of organisms for the study have been followed and all necessary approvals have been obtained.

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