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Description of a New Subfamily, Astrocloninae (Ophiuroidea: Euryalida: Gorgonocephalidae), Based on Molecular Phylogeny and Morphological Observations

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Previous phylogenetic studies of the family Gorgonocephalidae (brittle stars and basket stars) have identified three subfamilies, Astrotominae, Astrothamninae, and Gorgonocephalinae. The genus *Astroclon* was tentatively assigned to the subfamily Astrothamninae in previous studies, but its morphology is enigmatic and molecular data of the genus was insufficient. Therefore, the systematic position of *Astroclon* required confirmation to reconstruct the accurate systematics of the Euryalida. In the present study, we sought to clarify the subfamilial classification in the family Gorgonocephalidae (Echinodermata: Ophiuroidea: Euryalida). We revisited molecular phylogenetics of the Gorgonocephalidae including *Astroclon* and the major taxa in the family. The molecular analysis supported monophyly of the two species of *Astroclon* and its clear separation from Astrothamninae. The two *Astroclon* species were also distinguished from the other two subfamilies, Astrotominae and Gorgonocephalinae. *Astroclon* and three other subfamilies were divided in the basal position of the Gorgonocephalidae, and were assigned to subfamilies. A new subfamily, Astrocloninae was monotypically established for *Astroclon* in addition to the previous three subfamilies. Morphology of the first ventral arm plates and tentacle pores also supported distinctiveness of the new subfamily.

Key words: brittle stars, taxonomy, marine invertebrates, ventral arm plate, Japan

INTRODUCTION

Gorgonocephalidae is the largest family of the order Euryalida and currently includes 34 genera (Döderlein, 1927; Fell, 1960; Okanishi and Fujita, 2013; Okanishi, 2016). In a recent molecular study, the family was identified as monophyletic with three recognized subfamilies, Astrotominae, Gorgonocephalinae, and Astrothamninae (Okanishi and Fujita, 2013). The subfamilies were distinguished by the morphology of madreporites, teeth, and genital slits. The presence/absence of supplementary oral plates were also emphasized by Matsumoto (1917), however, the plates have only been observed for *Astrochele pacifica* (Mortensen, 1933), *Gorgonocephalus arcticus*, *Astrochele* sp., and *Astrotoma* sp. (Ezhova et al., 2016). Observation of supplementary oral plates requires destructive dissection of surficial skins and/or external ossicles in the oral area; thus, no other work has recorded the nature of this structure. As a result, Okanishi and Fujita (2013) did not take this character into account in their systematics.

The enigmatic and rare genus *Astroclon* was monotypically established for *Astroclon propugnatoris* Lyman, 1879 in Gorgonocephalidae by Lyman (1879) and later another species *A. suenisoni* Mortensen, 1911 was described (Mortensen,

1911). The former species has been recorded from Indonesia, Philippines, and Japan in the literature (Lyman, 1879; Mortensen, 1933; Murakami, 1944; Guille, 1981; Irimura and Kubodera, 1998; Okanishi et al., 2011) and the latter has been recorded from Australia and Japan (Mortensen, 1911, 1933; Baker, 1980). In the current systematics, *Astroclon* is classified as Astrothamninae in having a madreporite on the disc periphery as well as slit-shaped genital slits, and by the absence of oral papillae (Okanishi and Fujita, 2013).

Nuclear 28S rRNA, 18S rRNA, and mitochondrial 16S rRNA genes of *Astroclon propugnatoris* were sequenced by Okanishi et al. (2011). The phylogenetic tree of 49 species of Euryalida, including 27 gorgonocephalid species, showed *A. propugnatoris* was clearly included in Gorgonocephalidae. However, its exact systematic position was unclear (Okanishi et al., 2011). Okanishi and Fujita (2013) analyzed the phylogeny of 83 species of Euryalida including 38 gorgonocephalid species, mainly using mitochondrial COI, but *A. propugnatoris* was not included in this analysis (Okanishi and Fujita, 2013) and no material for sequencing of *A. suenisoni* has been obtained.

In this study, to confirm the systematic position of the genus *Astroclon* within the Gorgonocephalidae, we obtained nuclear 18S rRNA, and mitochondrial COI and 16S sequences of a fresh specimen of *A. suenisoni* collected from central Japan for the phylogenetic analysis. We also review important morphological characters including sup-

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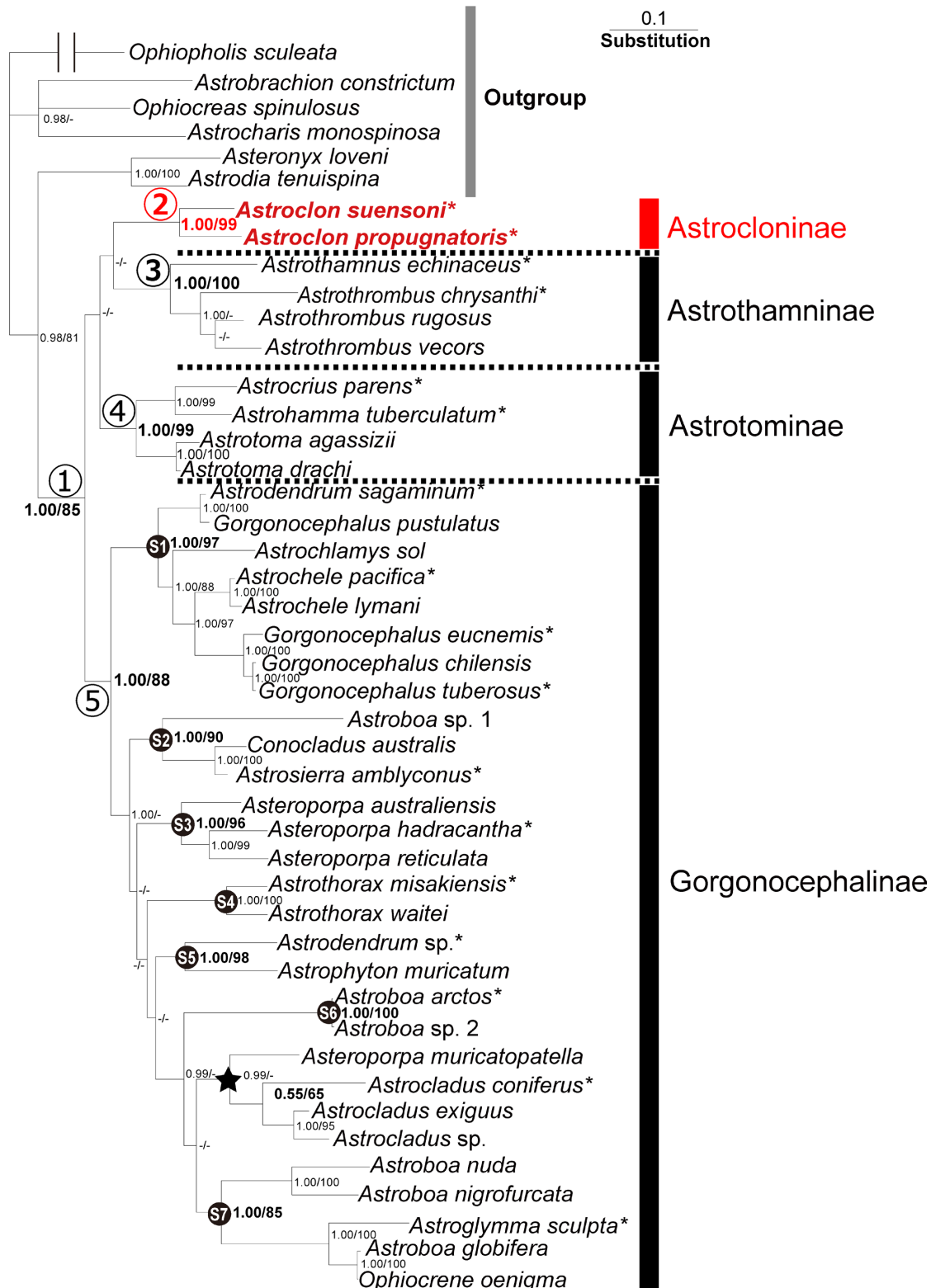


Fig. 1. Bayesian tree based on a partitioned analysis of the nuclear 18S rRNA, and mitochondrial COI and 16S rRNA and genes (2889 bp). *Ophiopholis aculeata* (Ophintegridae; Ophiactidae), *Astrobrachion constrictum* (Euryalidae, Euryalinae), *Astrocharis monospinosa* (Euryalidae, Astrocharinae), *Ophiocreas spinulosus* (Euryalidae, Asteroschematinae) and *Asteronyx loveni* and *Astrodia tenuispina* (Asteronychidae) were used as the outgroup. Support values for each node are shown by Bayesian posterior probabilities and maximum likelihood bootstrap values. Nodes in the phylogenetic trees were considered as supported when BPP and bootstrap values were larger than 0.98 and 85%, respectively. BPP values lower than 0.97 and bootstrap lower than 74% for each node were considered not significant and shown by a hyphen. Support value of a clade of *Astroclon* was shown since it is discussed in the text. Numerals (S1–S7) in circles and a star at nodes refer to the clade number discussed in the text. Morphology was observed in this study for species appended with as asterisks.

plementary oral plates of many gorgonocephalid species to compare them with those of other *Astroclon* species.

MATERIALS AND METHODS

Sample collections for molecular analysis

One specimen of *Astroclon suensoni* was collected by a fishing line, off Kashino-zaki, Kushimoto, Wakayama, central Japan, 100–140 m depth, 9 January 2014 (NSMT E-10717), and an arm tip specimen of *Astroclon propugnatoris* was collected by ROV Hakuyo 2000 of M/S Shinsei-maru (Fukada Salvage and Marine Works Co. Ltd.), off Tarama-jima Island, Okinawa, southwestern Japan, 24°29.72'N, 124°33.78'E. 284–290 m depth, 8 March, 2005 (NSMT E-6274). These two specimens were directly immersed in 99% ethanol for molecular analysis.

DNA extraction, PCR amplification, and DNA sequencing

We sequenced nuclear 18S rRNA and mitochondrial COI and

16S rRNA genes for *Astroclon suensoni* and mitochondrial COI for *Astroclon propugnatoris*. The method of DNA extraction and PCR parameters followed that by Okanishi and Fujita (2013). Total DNA solution of *Astroclon* was diluted by distilled water 50 times for PCR of 18S rRNA, 100 times for COI and 16S rRNA. Primer sets of SSU001 (5'-GCTTGTCTTAAAGACTAAGCCATGC-3') and SSU002 (5'-CCGTGTTGAGTCAAATTAAGCCGC-3') (Okanishi et al., 2011) was used for PCR of 18S rRNA, COI005 (5'-TTAGGTAAHWAAAC-CAVYTKCCTTCAAAG-3') and COI008 (5'-CCDTANGMDATCAT-DGCRATACATCATTC-3') (Okanishi and Fujita, 2013) was used for PCR of COI, and 16Sar (5'-CGCCTGTTTATCAAAAACAT-3') and 16Sbr (5'-CCGGTCTGAAGTCAATCAGATCACGT-3') (Palumbi, 1996) was used for PCR of 16S rRNA. The PCR products were separated from excess primers and oligonucleotides using Exo-SAP-IT (GE Healthcare), following the manufacturer's protocol. All samples were sequenced bidirectionally and sequence products were run on a 3730xl DNA Analyzer (Thermo Fisher Scientific). The accession numbers of DNA Data Bank of Japan (DDBJ) of the *Astroclon*

Table 1. Sampling locality and voucher depository of the examined specimens. Asterisks indicate type species.

Subfamilies and species	Locality	Museum Catalog Number
<i>Astrocloninae</i> subfam. nov.		
<i>Astroclon propugnatoris</i> Lyman, 1879*	East China Sea, Japan. 29°23.00'N, 127°14.06'E. 158–175 m depth	NSMT E-3564
<i>Astroclon suensoni</i> Mortensen, 1911*	Naze Ridge, Amami Trough, Japan. 28°34.46'N, 129°29.73'E. 429–432 m depth	NSMT E-10755
<i>Astrothamninae</i> Okanishi & Fujita, 2013		
<i>Astrothamnus echinaceus</i> Matsumoto, 1912*	Off Miyake-jima Isl., Japan. 33°52.02'N, 140°14.06'E. 470 m depth	NSMT E-2207-A
<i>Astrothrombus chrysanthi</i> Matsumoto, 1918	Off Otsuchi, Iwate, Japan. 39°21.86'N, 141°59.86'E. 78.2 m depth	NSMT E-6715
<i>Astrotominae</i> Matsumoto, 1915		
<i>Astrocrius parens</i> (Koehler, 1930)	SE of Boso Peninsula, Japan. 440–420 m depth	NSMT E-6897
<i>Astrohamma tuberculatum</i> (Koehler, 1923)	Ross Sea., Antarctica. 67°38.05'S, 165°38.00'E. 513 m depth	NSMT E-679
<i>Astrotoma manilense</i> Döderlein, 1927*	S of Yaku-shima Isl., Japan. 30°02.04'N, 130°57.03'E. 660–710 m depth	NSMT E-3148
<i>Gorgonocephalinae</i> Ljungman, 1867		
<i>Asteroporpa hadracantha</i> H. L. Clark, 1911	Takase Bank, Japan. 34°28.06'N, 139°12.08'E. 80–190 m depth	NSMT E-3053
<i>Asteroporpa annulata</i> Örsted & Lütken, 1856	E of Sebastian Inlet, Florida, USA.	NSMT E-1767
<i>Astroboa arctos</i> Matsumoto, 1915	Kushimoto, Wakayama, Japan. ca. 10 m depth	NSMT E-5644
<i>Astrochele pacifica</i> Mortensen, 1933	East China Sea, Japan. 26°16.46'N, 124°51.65'E. 1000–1240 m depth	NSMT E-1553
<i>Astrochlamys bruneus</i> Koehler, 1912	Ross Sea., Antarctica. 67°38.05'S, 165°38.00'E. 513 m depth	NSMT E-697
<i>Astrocladus coniferus</i> (Döderlein, 1902)	East China Sea, Japan. ca. 108–109 m depth	NSMT E-5339
<i>Astrodendrum sagaminum</i> (Döderlein, 1902)	East China Sea, Japan. Depth unknown	NSMT E-794
<i>Astrodendrum</i> sp.	Off Mie, Japan. 111 m depth	NSMT E-10715
<i>Astroglymma sculptum</i> (Döderlein, 1896)	Off Minabe, Wakayama, Japan. ca. 30 m depth	NSMT E-10718
<i>Astrosierra amblyconus</i> (H. L. Clark, 1909)	Servis Bay, New South Wales, Australia. 110 m depth	NSMT E-1943
<i>Astrothorax misakiensis</i> Döderlein, 1911	Off Hachijo-jima Isl., Japan. 33°09.00'N, 140°02.02'E. 475 m depth	NSMT E-2673
<i>Gorgonocephalus eucnemis</i> Müller and Troschel, 1842*	East China Sea, Japan. 28°37.14'N, 126°49.17'E. 195–220 m depth	NSMT E-1563
<i>Gorgonocephalus tuberosus</i> Döderlein, 1902*	Southern Chile. 45°04.S, 74°38.W. 74 m depth	NSMT E-7006
<i>Schizostella</i> sp.	Carrie Bow Cay, Belize. Depth unknown	NSMT E-1769

are LC272069 (18S rRNA), LC272067 (COI), and LC272070 (16S rRNA) for *A. suenisoni*, and LC272068 (COI) for *A. propugnatoris*.

Phylogenetic analysis

We analyzed sequences of both species of *Astroclon*, with the sequence data of other 38 gorgonocephalid species obtained by Okanishi and Fujita (2013). For outgroups, we selected five euryalid species with the shortest genetic distance from Gorgonocephalidae, two from Asteronychidae and three from Euryalidae, to avoid long branch attraction (Bergsten, 2005; Okanishi and Fujita, 2013). Additionally, we selected *Ophiopholis aculeata* (Ophintegrida; Ophiactidae) as a representative of Ophintegrida. This species is also used in previous molecular phylogeny (Okanishi and Fujita, 2013) with sequences of 18S rRNA, COI, and 16S rRNA genes.

All sequences were aligned using the Clustal W algorithm in MEGA5 (Thompson et al., 1994; Tamura et al., 2011). Gene regions where the alignment was ambiguous, including ribosomal loops, were excluded by eye, and all missing sequences were scored as gaps. Overall average of substitution rate was computed using MEGA5 according to the Kimura 2-parameter model (Kimura, 1980) to compare the evolutionary rate of each gene. The Kimura 2 parameter model (Kimura, 1980) with a gamma distribution was selected as best-fit model of 18S rRNA gene, and general time reversible model (GTR; Yang, 1994a) assumed to be invariable (I; Hasegawa et al., 1985) with a gamma distribution approximated by discrete categories (Γ; Yang, 1994b) being selected as the best-fit model of nucleotide substitution with COI and 16S rRNA genes, by using the "Find best fit models" option of MEGA5. BioEdit ver. 7.0.5.3. (Hall, 1999) and Seaview ver. 4.3.0 (Gouy et al., 2010) were used in preparing the data matrices in FASTA and PHYLIP format, respectively. Seaview ver. 4.3.0 was also used in concatenating sequences of three gene sequences. Tree View for Win 16 (Page, 1996) was used in exploring tree files, in preparing NEWICK format and exploring alternative tree topologies. The phylogenetic tree was constructed with MrBayes ver. 3.1.2 (Ronquist and Huelsenbeck, 2003) to obtain Bayesian posterior probabilities (BPP) and RAxML ver. 8.1.20 (Stamatakis, 2014) for maximum likelihood analysis (ML) to obtain bootstrap support values. The three gene sequences were placed in separate partitions. We set parameters in MrBayes as follows; two substitution types (nst = 2, statefreqpr = fixed; K2P) for 18S rRNA and six substitution types (nst = 6; GTR) for COI and 16S rRNA were employed; rate variation across sites was modeled using a gamma distribution (rate = gamma) for 18S rRNA and using a gamma distribution with a proportion of sites being invariant (rate = invgamma) for COI and 16S rRNA; the shape, proportion of invariable sites, state frequency, and substitution rate parameters were estimated for each partition separately. The Markov-Chain Monte-Carlo (MCMC) process was run with four chains for 4,000,000 generations, with trees being sampled every 100 generations. The first 10,000 trees were discarded as burn-in. Data sets were partitioned by gene region for the maximum likelihood analysis to allow for separate optimization per-site substitution rates. The best-supported likelihood tree was found by performing 1000 replications. Nodes in the phylogenetic trees were considered as supported if BPP values were larger than 0.98 and bootstrap value larger than 85%. BPP values lower than 0.97 and bootstrap lower than 74% for each node were considered as not significant (Fig. 1).

Morphological observation

For morphological comparison between the two species of *Astroclon* (*A. propugnatoris* and *A. suenisoni*) and other gorgonocephalids, 19 species in 16 genera covering the three subfamilies of Gorgonocephalidae were examined (Table 1). Specimens identifiable as the type genera of the three subfamilies, *Astrothamnus*, *Astrotoma*, and *Gorgonocephalus*, were included. From *Astrothamninae* and *Astrotominae*, we examined one or more species from each of the five genera (*Astrothamnus*, *Astrothrombus*, *Astrocris*,

Astrohamma, and *Astrotoma*). Since we did not obtain any dissectable specimens of *Astrotoma drachi* and *A. agassizii* (which were only used for obtaining molecular data), we examined a specimen of *A. manilense* as a representative of this genus; according to O'Hara and Harding (2014), *A. drachi* and *A. manilense* are potentially synonymous. In Gorgonocephalinae, some genera were recovered to be non-monophyletic (Okanishi and Fujita, 2013). Therefore, we selected species so that they represent all the gorgonocephaline subclades recognized by molecular analysis in this study (see Fig. 1, nodes S1–S7). We examined one species of *Astrochele*, one species of *Astrodendrum*, and two species of *Gorgonocephalus* from clade S1, and one species each from clades S2–S7 (*Astrosierra* from S2, *Asterporpa* from S3, *Astrothorax* from S4, *Astrodendrum* S5, *Astroboa* from S6, *Astroglymma* from S7).

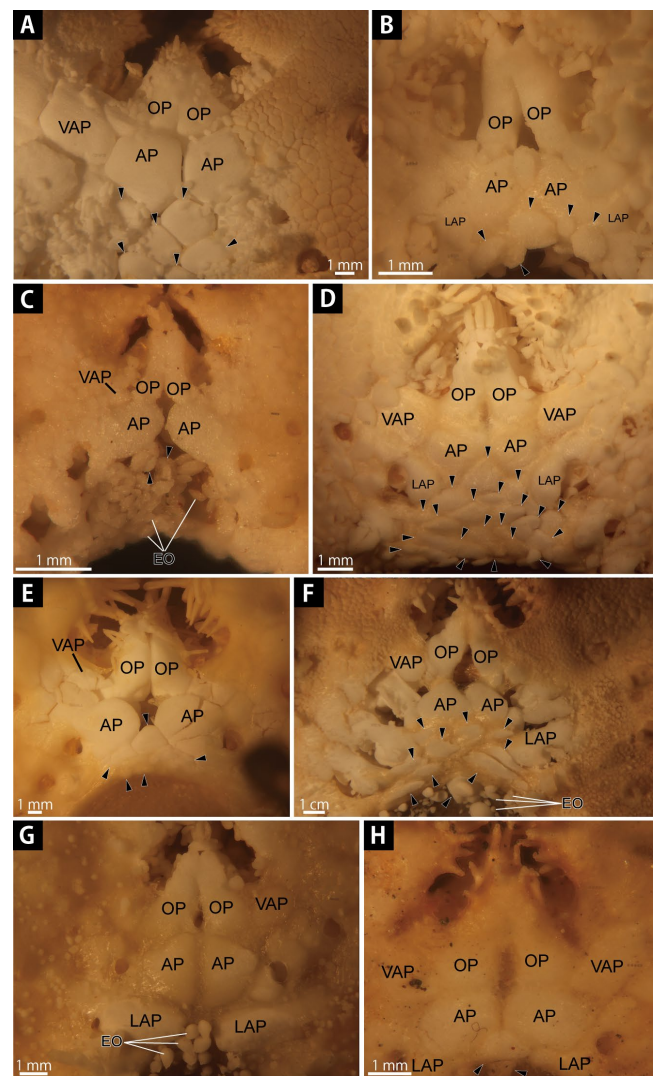


Fig. 2. Oral views of disc skins and external ossicles were partly removed. (A) *Astroclon suenisoni* (NSMT E-10755); (B) *Astrothamnus echinaceus* (NSMT E-2207-A); (C) *Astrothrombus chrysanthi* (NSMT E-6715); (D) *Astrotoma manilense* (NSMT E-3148); (E) *Gorgonocephalus eucnemis* (NSMT E-1563); (F) *Astrosierra amblyconus* (NSMT E-1943); (G) *Asteropora annulata* (NSMT E-1767); (H) *Astrochlamys bruneus* (NSMT E-697). Black arrowheads indicate supplementary oral plates. Abbreviations: AP, adoral plate; EO, external ossicle; LAP, lateral arm plate; OP, oral plate; VAP, ventral arm plate.

Because *Asteropora muricatopatella* and three species of *Astrocladus* formed a clade despite with the relatively lower support value of ML, we selected *Astrocladus coniferus* as a representative of this species group (see Fig. 1, node marked by a star).

In morphological observation, we focused on supplementary oral plates, adoral plates, and the first ventral arm plates, all recognized as diagnostic characters of subfamilies in Gorgonocephalidae since Matsumoto (1917). The presence/absence of supplementary oral plates have been observed only for *Astrocrius sobrina* (Astrotominae) (Matsumoto, 1917). Adoral plates and the first ventral arm plates have been observed for a limited number of species (e.g., *Astrochele pacifica*, Mortensen, 1933), but never for *Astroclon*. We also examined the following external characters used in generic diagnoses in Gorgonocephalidae: presence/absence of tubercles, spines, special calcareous plates, and concentric transverse ridges on disc; maximum number of arm spines; maximum numbers of secondary teeth on hooklets; and patterns of arm branching (see Matsumoto, 1917; Döderlein, 1927; Mortensen, 1933; Fell, 1960; Turner and Boucher, 2010).

To observe the supplementary oral plates, adoral plates, and the first ventral arm plates, skin and external ossicles were removed using domestic bleach (~5% sodium hypochlorite solution). The aboral plates and the first ventral arm plates were isolated, washed in deionized water, observed and photographed with Keyence VHX 600. The terms used in this study to describe brittle stars follow Matsumoto (1917), Martynov (2010), Stöhr et al. (2012), Okanishi

and Fujita (2013), and Okanishi (2016). All the specimens with isolated ossicles are deposited in the National Museum of Nature and Science, Japan (NSMT).

RESULTS

Molecular phylogeny

After removal of ambiguously aligned sites, we were left with 949 bp of 18S rRNA, 1511 bp of COI, and 429 bp of 16S rRNA. Overall averages of nucleotide substitution rates within nuclear 18S rRNA, mitochondrial COI and 16S rRNA genes were 0.019, 0.261, and 0.186, respectively.

The Bayesian tree of concatenated sequences of the three genes, 18S rRNA, COI, and 16S rRNA is shown in Fig 1. The ML tree also showed the same topology. Both Bayesian and ML analyses supported monophyly of Gorgonocephalidae (Fig. 1, node 1, bootstrap value 85%, BPP 1.00).

Two species of *Astroclon* formed a clade (Fig. 1, node 2, bootstrap 99%, BPP 1.00). The Gorgonocephalidae clade was divided into the following four clades: two species of *Astroclon* (Fig. 1, node 2, bootstrap 99%, BPP 1.00), *Astrothamninae* (Fig. 1, node 3, bootstrap 100%, BPP 1.00), *Astrotominae* (Fig. 1, node 4, bootstrap 99%, BPP 1.00), and *Gorgonocephalinae* (Fig. 1, node 5, bootstrap 88%, BPP 1.00). In Gorgonocephalinae, monophyly of seven sub-

Table 2. Morphological characters of examined species. Abbreviations: AS, arm spine; SOP, supplementary oral plate; SCP, special calcareous plate; ST, secondary teeth of hooklet; VAP, ventral arm plate. Adoral plates are not included because no morphological differences were observed between species.

Subfamilies and species	Disc					Arm			
	Ornaments	Concentric transverse ridge	SCP on marginal edge	Number of SOP	Wedge-shaped projection of 1st VAP	Patterns of arm branching	Maximum number of ST	Pits of tentacle pore	Maximum number of AS
Astrocloninae subfam. nov.									
<i>Astroclon propugnatoris</i>	Absent	Absent	Absent	6	Present	Branching arm tip	3	Present	6
<i>Astroclon suensoni</i>	Tubercles	Absent	Absent	6	Present	Branching arm tip	3	Present	6
Astrothamninae									
<i>Astrothamnus echinaceus</i>	Spines	Absent	Absent	5	Absent	Simple, not branching	6	Absent	3
<i>Astrothrombus chrysanthi</i>	Tubercles	Absent	Absent	5	Absent	Simple, not branching	3	Absent	2
Astrotominae									
<i>Astrocrius parens</i>	Tubercles	Absent	Absent	17	Absent	Simple, not branching	2	Absent	3
<i>Astrohamma tuberculatum</i>	Tubercles	Absent	Absent	3	Absent	Simple, not branching	1	Absent	3
<i>Astrotoma manilense</i>	Spines	Absent	Absent	20	Absent	Simple, not branching	Absent	Absent	3
Gorgonocephalinae									
<i>Asteropora hadracantha</i>	Absent	Absent	Absent	1	Absent	Simple, not branching	1	Absent	5
<i>Asteropora annulata</i>	Absent	Absent	Absent	Absent	Absent	Simple, not branching	1	Absent	6
<i>Astroboa arctos</i>	Absent	Absent	Absent	15	Absent	Branching from basal arm	1	Absent	2
<i>Astrochele pacifica</i>	Absent	Absent	Absent	5	Absent	Simple, not branching	3	Absent	5
<i>Astrochlamys bruneus</i>	Absent	Absent	Absent	1	Absent	Simple, not branching	0	Absent	4
<i>Astrocladus coniferus</i>	Tubercles	Absent	Absent	30	Absent	Branching from basal arm	1	Absent	3
<i>Astrodendrum sagaminum</i>	Absent	Absent	Absent	4	Absent	Branching from basal arm	1	Absent	3
<i>Astrodendrum</i> sp.	Absent	Absent	Absent	4	Absent	Branching from basal arm	2	Absent	3
<i>Astroglymma sculptum</i>	Absent	Absent	Absent	5	Absent	Branching from basal arm	1	Absent	1
<i>Astrosierra amblyconus</i>	Tubercles and spines	Absent	Absent	10	Absent	Branching from middle arm	1	Absent	5
<i>Astrothorax misakiensis</i>	Absent	Absent	Absent	4	Absent	Simple, not branching	1	Absent	6
<i>Gorgonocephalus eucnemis</i>	Absent	Absent	Present	4	Absent	Branching from basal arm	1	Absent	4
<i>Gorgonocephalus tuberosus</i>	Spines	Absent	Present	13	Absent	Branching from basal arm	1	Absent	5
<i>Schizostella</i> sp.	Absent	Present	Absent	Absent	Absent	Branching arm tip	Absent	Absent	2

clades were well supported (Fig. 1, node S1–S7, bootstrap 85–100%, BPP 1.00), whereas support value of a subclade of three species of *Astrocladus* and *A. muricatopatella* was lower in our ML analysis (Fig. 1, node star).

Morphological observation

Supplementary oral plates were present on 19 species including *Astroclon* but were lacking on *Asteropora*

annulata Örsted and Lütken, 1856 (in Lütken, 1856) and *Schizostella* sp. (Fig. 2; Table 2).

Adoral plates were flat and plate-shaped for all examined species, with no distinct morphological differences being confirmed between *Astroclon* and the other gorgonocephalids. However, the first ventral arm plates were different in shape between *Astroclon* and the other gorgonocephalids. The oral surface of the first ventral arm plates of

Astroclon propugnatoris (Fig. 3A) and *A. suenisoni* were polygonal and flat, with the wedge-like projections protruding from the aboral surface (Fig. 3A). The first ventral arm plates of the other 19 gorgonocephalid species were plate-shaped, and both oral and aboral surfaces were polygonal and flat with no projections (Fig. 3B–D; Table 2).

Tentacle pores of *Astroclon propugnatoris* and *A. suenisoni* had a large pit beside tentacle sheath (Fig. 4A), while no such pit was observed for the other 19 gorgonocephalid species (Fig. 4B–D).

We observed no distinctive features for *Astroclon* in the six external characters used as generic diagnoses in Gorgonocephalidae: presence/absence of tubercles, spines; presence/absence of special calcareous plates; presence/absence of concentric transverse ridges on disc; maximum number of arm spines; maximum number of secondary teeth on hooklets; and patterns of arm branching (Table 2).

DISCUSSION

In the molecular tree of Okanishi and Fujita (2013), three clades were recognized in Gorgonocephalidae and classified as three subfamilies, Astrothamninae (including *Astroclon*), Astrotominae, and Gorgonocephalinae. In the present study, the two species of *Astroclon* were distinctly separated from the other genera of Astrothamninae and formed a clade (Fig. 1). The *Astroclon* was also separated from Astrotominae and Gorgonocephalinae clades, and monophyly of the two subfamilies and Astrothamninae, except *Astroclon*, was well supported. The *Astroclon* and the three subfamily clades were diverged near the base of the family Gorgonocephalidae. Branch length between the *Astroclon* and Astrothamninae was longer than those between Astrothamninae and Astrotominae, and between Astrothamninae and Gorgonocephalinae. These molecular evidences suggest that *Astroclon* is well genetically separated from other three subfamilies and the systematic position of *Astroclon* should be elevated to the same rank as the three subfamilies.

By our morphological observations,

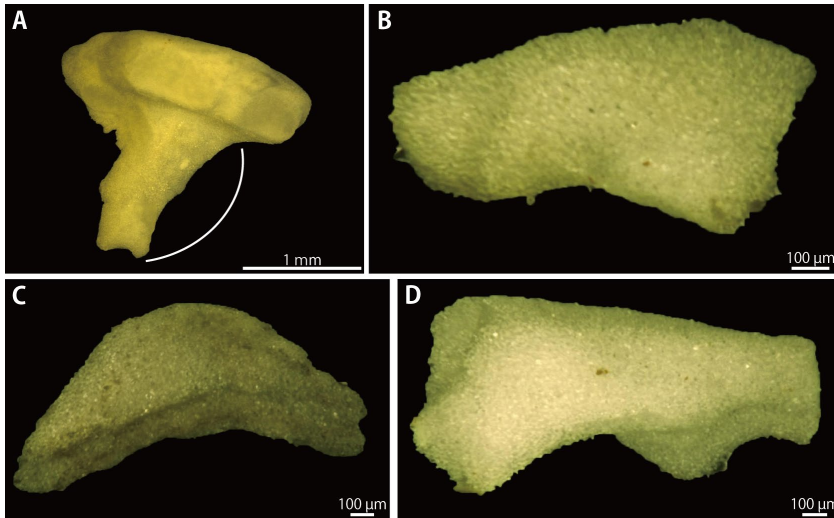


Fig. 3. Lateral view of first ventral arm plate. (A) *Astroclon suenisoni* (NSMT E-10755); (B) *Astrothamnus echinaceus* (NSMT E-2207-A); (C) *Astrotoma manilense* (NSMT E-3148); (D) *Astroglymma sculptum* (NSMT E-10718). Orientations: top oral side and bottom aboral side. Arc indicates wedge-like structure.

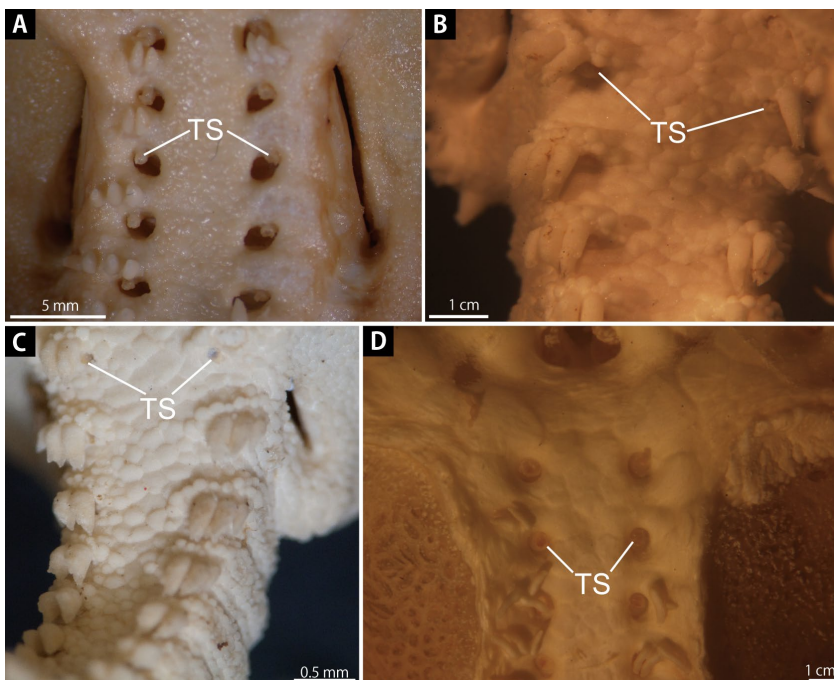


Fig. 4. Oral view of basal portion of an arm. (A) *Astroclon propugnatoris* (NSMT E-3564); (B) *Astrothamnus echinaceus* (NSMT E-2207-A); (C) *Astrotoma manilense* (NSMT E-3148); (D) *Gorgonocephalus eucnemis* (NSMT E-1563). (A) large pits present adjacent to tentacle sheath; (B–D) no pits adjacent to the sheath. Abbreviation: TS, tentacle sheath.

Table 3. Diagnostic characters distinguishing four subfamilies of Gorgonocephalidae in the proposed classification.

Subfamilies	Wedge-shaped projections of the first ventral arm plates	Oral papillae	Position of madreporite	Shape of genital slits	Pits of tentacle pores
Astrocloninae subfam. nov.	Present	Absent	Peripheral	Slit	Present
Astrothamniinae	Absent	Rudimentary/absent	Peripheral	Slit	Absent
Astrotominae	Absent	Rudimentary/absent	Oral	Slit	Absent
Gorgonocephalinae	Absent	Spiniform	Peripheral	Pore	Absent

previously used taxonomic characters by Matsumoto (1917) did not distinguish the three subfamilial clades and *Astroclon* recognized by our molecular analysis. Presence/absence of supplementary oral plate was considered to distinguish Matsumoto (1917)'s Gorgonocephalinae (=current Gorgonocephalinae) and Astrotominae (= current Astrotominae + Astrothamniinae + *Astroclon*) in Gorgonocephalidae; however, they were absent only in *Asteropora annulata* and *Schizostella* sp. of Gorgonocephalinae. The six generic characters mentioned above also did not distinguish the subfamilial clades (Table 2). Therefore, traditionally known morphological characters have been confirmed unavailable for subfamilial taxonomy of Gorgonocephalidae.

The *Astroclon* was originally classified in Astrothamniinae which shared the following morphological characters: a madreporite located on periphery disc, slit-like genital slits, and absence of oral papillae (Okanishi and Fujita, 2013). Our morphological observations newly showed that *Astroclon* has a unique wedge-shaped projection on the first ventral arm plate (Fig. 3A) while the other examined genera have no such projections (Fig. 3B–D; Table 2), and has large pits adjacent to tentacle sheath (Fig. 4A) which are also lacking in the other examined genera (Fig. 4B–D). Although the shapes of the first ventral arm plates have not been described previously, our literature surveys for descriptions of tentacle pores of all gorgonocephalid species found that large pits were only possessed by *Astroclon* in Gorgonocephalidae (Linnaeus, 1758; Retzius, 1783; Lamarck, 1816; Leach, 1819; Risso, 1826; Müller and Troschel, 1842; Duchassaing, 1850; Lütken, 1856; Philippi, 1858; Lyman, 1861, 1869, 1874, 1875, 1879; Verrill, 1867, 1876, 1878, 1899; Bell, 1894; Döderlein, 1896, 1898, 1902, 1911, 1927, 1930; Koehler, 1897, 1898, 1904, 1905, 1910, 1912, 1922, 1923, 1930; Lütken and Mortensen, 1899; Benham, 1909; H. L. Clark, 1909, 1911, 1914, 1915, 1938; Mortensen, 1911, 1912, 1933, 1936; Matsumoto, 1912, 1915, 1918; A. H. Clark, 1916, 1919, 1948, 1952; Guille, 1979; Baker, 1980; McKnight, 2000; Okanishi and Fujita, 2011). *Astroclon* is clearly distinguished morphologically not only from the other genera of Astrothamniinae, but also from Astrotominae and Gorgonocephalinae. Therefore, our comprehensive morphological study also supports treating the *Astroclon* and the three subfamilies as equally ranked.

Combination of the newly found two unique characters, presence/absence of wedge-shaped projections of the first ventral arm plate and pits besides tentacle pore, by our study (Figs. 3, 4), and previously used three subfamilial characters, shapes of oral papillae, position of madreporite, and shape of genital slits, found by Okanishi and Fujita

(2013), clearly distinguishes the genus *Astroclon* from the three subfamilies in Gorgonocephalidae (Table 3). Therefore, these four clades in a multi-branching relationship (Fig. 1) can be classified as four subfamilies with distinct different morphological characters, and we propose a new subfamily, Astrocloninae, monotypic for the genus *Astroclon*. The systematic position and definition of the new subfamily are as follows:

Order **Euryalida** Lamarck, 1816
Family **Gorgonocephalidae** Ljungman, 1867
Subfamily **Astrocloninae** subfam. nov.

Diagnosis. Gorgonocephalids with a madreporite located on disc periphery; slit-shaped genital slits, absence of oral papillae; tentacle pores with large pits and wedge-shaped first ventral arm plates.

Type genus. *Astroclon* Lyman, 1879

Other included genera. none.

Distribution. Australia: off Broome (Baker, 1980). Indonesia: Arafura Sea (Lyman, 1879), off Manado (Mortensen, 1933). Philippines: off Lubang Islands (Guille, 1981). Japan: off Goto Island (Mortensen, 1911, 1933), East China Sea (Irimura and Kubodera, 1998), off Okinawa Island (Okanishi et al., 2011), off Amami and Kushimoto (this study). The bathymetric range is 158–457 m.

Remarks. Astrocloninae is clearly distinguished from the other three subfamilies of Gorgonocephalidae by five morphological characters (Table 3). The monotypic genus *Astroclon* includes *A. propugnatoris* Lyman, 1879 (type species) and *A. suenonni* Mortensen, 1911.

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COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

MO performed molecular phylogenetic analysis, reconstruction of molecular tree, morphological observations of specimens. Manuscript including figures and Tables were prepared by MO and brush upped by MO and TF.

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