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A New Species of Sexually Dimorphic Brittle Star of the Genus *Ophiodaphne* (Echinodermata: Ophiuroidea)

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We describe a new species of sexually dimorphic brittle star, *Ophiodaphne spinosa*, from Japan associated with the irregular sea urchin, *Clypeaster japonicus* based on its external morphology, and phylogenetic analyses of mitochondrial COI (cytochrome c oxidase subunit I). Females of this new species of *Ophiodaphne* are characterized mainly by the presence of wavy grooves on the surface of the radial shields, needle-like thorns on the oral skeletal jaw structures, and a low length-to-width ratio of the jaw angle in comparison with those of type specimens of its *Ophiodaphne* congeners: *O. scripta*, *O. materna*, and *O. formata*. A tabular key to the species characteristics of *Ophiodaphne* is provided. Phylogenetic analyses indicate that the new species of *Ophiodaphne*, *O. scripta*, and *O. formata* are monophyletic. Our results indicate that the Japanese *Ophiodaphne* include both the new species and *O. scripta*, and that there are four *Ophiodaphne* species of sexually dimorphic brittle stars with androphorous habit.

Key words: sexually dimorphic ophiuroid, pseudocopulation, external morphology, molecular phylogenetic analyses, DNA barcode

INTRODUCTION

Four species of sexually dimorphic ophiuroids, the males of which grasp the larger female and press the ventral surface against that of the female, have been reported previously in Amphiuridae: *Ophiosphaera insignis* Brock, 1888, *Ophiodaphne scripta* (Koehler, 1904), *Ophiodaphne formata* (Koehler, 1905), and *Ophiodaphne materna* Koehler, 1930 (Brock, 1888; Koehler, 1930; Cherbonnier and Guille, 1978; Guille, 1981). *Ophiosphaera insignis* is easily distinguishable morphologically from its *Ophiodaphne* congeners by its dark-pigmented skin (Clark and Rowe, 1971). *Amphilycus androphorus* was first reported by Mortensen (1933), and subsequently synonymized with *O. scripta* by Clark (1967). These ophiuroids are not hermaphrodites, but dioecious species. Stöhr (2001) reported that *Amphipholis linopneusti* Stöhr, 2001 showed sexual dimorphism, and was epizoic on echinoids. However, it remains unknown whether it exhibits pseudocopulatory behaviors. In addition, *Ophiodaphne impellera* was recently described, but the report lacked descriptions of sexual dimorphism, reproductive biology, or host species (O'Hara and Harding, 2015).

Distributions of sexually dimorphic ophiuroids with androphorous habit range from Suez to the Indo-west Pacific Ocean, including Mozambique (*O. scripta*: Koeler, 1904; Cherbonnier and Guille, 1978; Parameswaran et al., 2013), from Gulf of Tonkin to New Caledonia, including west Australia (*O. formata*: Koehler, 1905; Clark, 1938; Guille, 1981; Guille and Vadon, 1986; Liao and Clark, 1995), from the southwestern coast of the Arabian peninsula to southeastern Polynesia (*O. materna*: Koehler, 1930; Clark, 1938, 1939, 1946; Clark and Rowe, 1971; Devaney, 1974). These records suggest that these three *Ophiodaphne* species are broadly distributed and that their distributions partly overlap. We cannot rule out the possibility that some specimens listed in the literature may be incorrectly identified (Fig. 1).

Androphorous dimorphic ophiuroids have a few uncommon characteristics compared to non-dimorphic ophiuroids. They are epizoic on the surface of their hosts, which are regular or irregular sea urchins and feather stars, and the females and males are sometimes coupled (Devaney, 1974; Clark and Courtman-Stock, 1976; Cherbonnier and Guille, 1978). They exhibit an unusual behavior that is considered pseudocopulation, and males are conspicuously dimorphic and small. Solitary (non-coupled) female and male sexually dimorphic ophiuroids are characterized as engaging in pseudocopulation compared with common ophiuroids.

Tominaga et al. (2004) reported the entire process of development of *O. scripta*. Its developmental mode is of the indirect type, with an ophiopluteus larva that spontaneously spawns in summer. Spawning behavior has not been observed between solitary females and males, but rather

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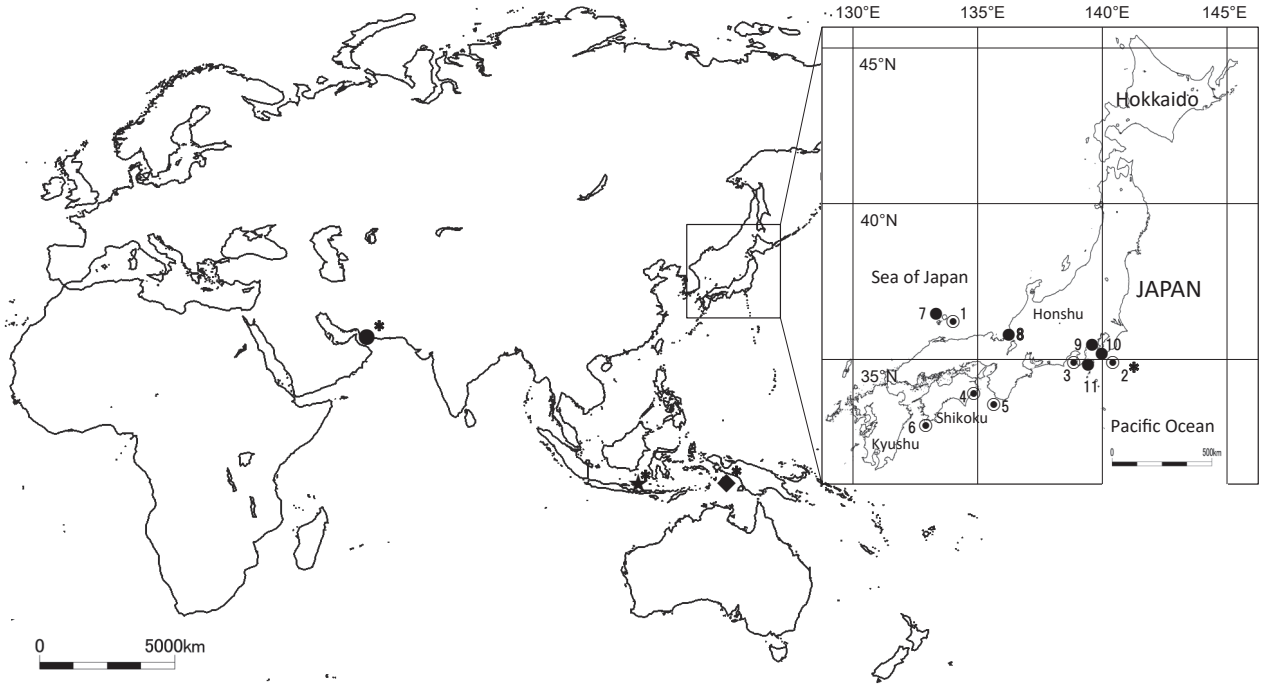


Fig. 1. Collection sites of sexually dimorphic brittle stars of *Ophiodaphne* congeners. Asterisks show type localities. ⊙ *Ophiodaphne spinosa* sp. nov.: 1 Oki Islands, 2* Tateyama Bay, 3 Iwachi, 4 Kaiyo, 5 Tanabe Bay, 6 Otsuki, Japan. ● *O. scripta*: 7 Oki Islands, 8 Tsuruga Bay, 9 Koajiro Bay, 10 Tateyama Bay, 11 Nabeta Bay, Japan. ●* *O. scripta*: Oman. ★* *O. formata*: Sumbawa Island, Indonesia. ◆* *O. materna*: Kei Island, Indonesia.

occurs during pseudocopulation (Tominaga et al., 2004). The pseudocopulatory behavior between females and males, which is indispensable for the reproduction of this ophiuroid, is followed by spawning. In the process of pseudocopulation of *O. scripta*, a solitary male approaches a female, quickly inverts, and clasps its mouth against that of a female (Tominaga and Komatsu, 2013). Coupled females and solitary individuals of both sexes of *O. scripta* always hold their dorsal surface against the surface of the host. They are furthermore capable of changing their position on a host, and transferring from one host to another (Tominaga and Komatsu, 2013). Reports of these developmental, behavioral, and ecological features have gradually elucidated the life history of sexually dimorphic ophiuroids. However, neither molecular data nor host interactions were previously available to enable the phylogenetic analyses of the genus *Ophiodaphne*. There have however been many taxonomic revisions and substantial confusion among three ophiuroid species: *O. scripta*, *O. formata*, and *O. materna*, which are superficially very similar (Koehler, 1904, 1905, 1930; Clark, 1938; Clark, 1967; Cherbonnier and Guille, 1978; Guille, 1981; Guille and Vadon, 1986; Parameswaran et al., 2013; Stöhr et al., 2015). Morphological interspecific variation of these sexually dimorphic ophiuroids has not been examined sufficiently, due to a lack of sufficient specimens.

We collected large numbers of sexually dimorphic ophiuroids with their hosts from Japan, and examined intra- and interspecific variations. Sexually dimorphic ophiuroids associated with the irregular sea urchin, *Astriclypeus manni* Verrill, 1867, from Japan appeared to be *O. scripta* (Koehler, 1904), although these had previously been misidentified as *O. formata* (Koehler, 1905) because of morphological similarities (Tominaga et al., 2004; Tominaga and Komatsu, 2013). Furthermore, the existence of another species was suspected associated with the host, *Clypeaster japonicus* Döderlein, 1885, from Japan, which was morphologically distinct from *O. scripta*.

In this study, we identify Japanese *Ophiodaphne* specimens and describe a new species based on external morphological comparisons with types, including observations by scanning electron microscopy and molecular analyses. A database of sexually dimorphic ophiuroids has recently been registered in GenBank by Hugall et al. (2016), contributing to molecular studies of this taxon. We performed a phylogenetic analysis based on mitochondrial COI partial sequences. Given our results, we seek to re-examine the classification of *Ophiodaphne* congeners.

MATERIALS AND METHODS

Collection sites and materials

New species of *Ophiodaphne* and *O. scripta* on their echinoid hosts, *Clypeaster japonicus* and *Astriclypeus manni*, respectively, were collected by scuba diving and dredging from shallow waters (≤ 20 m) in Japan. Samples in this study consisted of 145 females and 196 males of the newly identified species of *Ophiodaphne* (Tateyama Bay, Chiba Prefecture, 34°58′N, 139°47′E, 6–12 m; Iwachi, Shizuoka Prefecture, 34°44′N, 138°45′E, 8.2 m; Tanabe Bay, Wakayama Prefecture, 33°41′N, 135°20′E, 5 m; Kaiyo, Tokushima Prefecture, 33°32′N, 134°19′E, 5 m; Oki Islands, Shimane Prefecture, 36°09′N, 133°14′E, 1–19 m; Otsuki, Kochi Prefecture, 32°47′N, 132°41′E, 8–16 m), and 167 females and 161 males of *O. scripta* (Tateyama Bay, Chiba Prefecture, 34°58′N, 139°47′E, 6–12 m; Koajiro Bay, Kanagawa Prefecture, 35°09′N, 139°36′E, 10 m; Nabeta Bay, Shizuoka Prefecture, 34°39′N, 138°56′E, 10 m; Tsuruga Bay, Fukui Prefecture, 35°44′N, 136°03′E,

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5 m; Oki Islands, Shimane Prefecture, 36°09′N, 133°14′E, 20 m) (Fig. 1).

The type specimens, *Ophiodaphne scripta* (Koehler, 1904): syntype EcOh10661 deposited in the Museum National d'Histoire Naturelle (MNHN), *O. formata* (Koehler, 1905): holotype ZMA. V. ECH. O. 2006 deposited in the Naturalis Biodiversity Center (NBC), and *O. materna* Koehler, 1930: syntypes OPH–16 and OPH–17 deposited in the Zoological Museum University of Copenhagen (ZMUC) were used for identification of Japanese *Ophiodaphne* species. The holotype and paratypes of new species of *Ophiodaphne* used in this study are deposited in the National Museum of Nature and Science, Tsukuba, Japan (NSMT). DNA was extracted from specimens of the new species collected from Tateyama Bay, Iwachi, Kaiyo, and Otsuki; and *O. scripta* collected from Tateyama Bay, Koajiro Bay, Nabeta Bay, and Tsuruga Bay.

Morphological observations

Ophiuroids were removed from their hosts, fixed and preserved in 70% ethanol, and dried for microscopic observation. The following external characteristics were examined: degree of divergence of radial shields, shape of the teeth, the number of arm spines on the basal arm joints, ornamentation of disc scales on the dorsal disc, and the shapes of arm spines. For detailed observations of grooves on radial shields and disc scales, jaw ossicles (oral plates, adoral plates, and oral shield), papillae, and needle-like thorns at the outer edge of the jaw ossicles, dried material was coated with gold-palladium and examined using a Hitachi TM-1000 scanning electron microscope. The following measurements were taken using a stereomicroscope with an ocular micrometer: disc diameter (dd), as the distance from the base of the arm to the opposite margin of the disc on the dorsal surface; the length/width ratio of the "jaw angle" as defined by Clark (1967: fig. 2b) showing the distance between the proximal edge of the oralmost tooth and the distal medial edge of the oral shield, and maximum distance between the abradial edges of the paired adoral shields (Table 1).

DNA extraction, PCR amplification, and sequencing

Tissue samples of *O. spinosa* sp. nov. and *O. scripta*, were preserved in 99.5% ethanol at –30°C. Mouth, including muscle, was dissected under a stereomicroscope, and genomic DNA was extracted using a DNeasy Tissue Kit (Qiagen) according to the manufacturer's protocol. PCR of the partial COI gene was performed using the primers AsteroCOIf (TTCWACAAAWCATAAGGATATWGGAAC) (designed in this study) and COIceR (TCGTGTGTCTACGTCCATTCCTACTGTRAACATRTG) (Hoareau and Boissin, 2010) under the following conditions: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 47°C for 30 s, and 72°C for 2 min, with a final extension at 72°C for 7 min. Sequencing reactions using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and sequencing were performed on an ABI 3730XL DNA Analyzer (Applied Biosystems) by Eurofins Genomics (Tokyo, Japan). The sequences were deposited in GenBank, the European Molecular Biology Laboratory (EMBL), and the DNA Data Bank of Japan (DDBJ) under the Accession Numbers provided in Table 2.

Phylogenetic analysis

Sequence data of two specimens of *O. formata* (Koehler, 1905) were accessed through Barcode of Life Data Systems (BOLD: Ratnasingham and Hebert, 2007), which is managed by the Consortium for the Barcode of Life (Washington, DC). Licenses for the specimens are held by T. O'Hara (Museum Victoria, Melbourne, Australia). One sequence of *O. formata* (Accession No. KU895055) and *O. scripta* (KU895056) were cited for phylogenetic analysis. Four species of Amphiuridae (*Amphioplus andreae* KU894999; *Amphipholis linopneusti* KU895011; *Amphipholis squamata* KU895010; *Amphiura octacantha*, KU895025) were used as outgroups (Table 2).

Initial alignments were performed using MUSCLE (Edgar, 2004). The aim of our molecular analysis was "DNA barcoding" of *Ophiodaphne* spp., not to reveal the details of *Ophiodaphne* phylogeny. The following analysis used all three codon positions, although a saturation test indicated that the third codon position was saturated (data not shown). Pairwise base differences were calculated using MEGA ver. 5.2 (Kimura, 1980; Tamura et al., 2011). Maximum-likelihood (ML) analyses using RAxMLGUI ver. 1.3.1 (Silvestro and Michalak, 2012) and maximum-parsimony (MP) anal-

Table 1. Comparison of the morphological characteristics of four congeners of *Ophiodaphne* specimens. [a]Values in mm. Asterisks mean average. [b]Grooves on the radial shields and dorsal disc scales. [c]Thorns on the jaw ossicles. [d]Ratio of length to width of one jaw angle. NA; no data available.

Species	Locality	Disc diameter[a]		Radial shields	Groove[b]	Thorns[c]	Teeth	Ratio[d]	Arm spines	
		♀	♂	♀	♀	♀	♀	♀	♀	♂
Ophiodaphne spinosa sp. nov. E-7981, E-7982 (NSMT)	Tateyama Bay Japan	4.00	1.04	contiguous	present	present	pointed	1.12	6–7	3–4
Ophiodaphne spinosa sp. nov. non-type specimens	Japan	3.90* (*n* = 145; 1.00–5.50)	0.98* (*n* = 196; 0.44–1.36)	contiguous	present	present	pointed	1.15* (*n* = 44; 1.00–1.42)	6–7	3–4
Ophiodaphne scripta (Koehler, 1904) EcOh 10661 (MNHN)	Persian Gulf Oman	3.10	0.96	contiguous	present	absent	wide and blunt	1.40	4–5	3
Ophiodaphne scripta (Koehler, 1904) non-type specimens	Japan	4.14* (*n* = 167; 1.10–6.70)	0.86* (*n* = 161; 0.48–1.56)	contiguous	present	absent	wide and blunt	1.52* (*n* = 31; 1.28-1.82)	4–5	3
Ophiodaphne materna Koehler, 1930 OPH-17 (ZMUC)	Kei Is. Indonesia	4.33* (*n* = 4; 3.20–5.20)	1.36* (*n* = 2; 1.32–1.40)	contiguous	absent	absent	pointed	1.35* (*n* = 4; 1.23–1.43)	5–6	4
Ophiodaphne formata (Koehler, 1905) ZMA.V. ECH. O. 2006 (NBC)	Sumbawa Is. Indonesia	12.5	NA	divergent	absent	absent	pointed	1.22	4–5	NA

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Table 2. List of sequences of part of the mitochondrial COI genes from Amphiuridae species included in the phylogenetic analyses in this study. Sex*: F, female; M, male.

Species	Sex*	Host	Collection site	Abbreviation	COI	
					Accession No.	Ref.
Amphioplus andreae			Papua New Guinea	*A. andreae*	KU894999	Hugall et al. (2016)
Amphipholis linopneusti			New Caledonia	*A. linopneusti*	KU895011	Hugall et al. (2016)
Amphipholis squamata			USA	*A. squamata*	KU895010	Hugall et al. (2016)
Amphiura octacantha			Australia	*A. octacantha*	KU895025	Hugall et al. (2016)
Ophiodaphne formata			Australia	*O. formata*	KU895055	Hugall et al. (2016)
Ophiodaphne formata			Australia	*O. formata*_TOH-579	ECH0Z104-09	BOLD SYSTEM
Ophiodaphne formata			Australia	*O. formata*_TOH-682	ECH0Z017-09	BOLD SYSTEM
Ophiodaphne scripta			New Caledonia; -285M	*O. scripta*	KU895056	Hugall et al. (2016)
Ophiodaphne scripta	F	*Astriclypeus manni*	Tateyama, Chiba; -11.5M	*O. scripta*_Tateyama5F	LC091374	This study
Ophiodaphne scripta	M	*Astriclypeus manni*	Tateyama, Chiba; -11.5M	*O. scripta*_Tateyama6M	LC091375	This study
Ophiodaphne scripta	F	*Astriclypeus manni*	Tateyama, Chiba; -7M	*O. scripta*_Tateyama9F	LC091376	This study
Ophiodaphne scripta	M	*Astriclypeus manni*	Tateyama, Chiba; -7M	*O. scripta*_Tateyama10M	LC091377	This study
Ophiodaphne scripta	M	*Astriclypeus manni*	Tsuruga-Bay, Fukui	*O. scripta*_TsurugaM	LC091378	This study
Ophiodaphne scripta	F	*Astriclypeus manni*	Nabeta, Shizuoka	*O. scripta*_NabetaF	LC091379	This study
Ophiodaphne scripta	M	*Astriclypeus manni*	Nabeta, Shizuoka	*O. scripta*_NabetaM	LC091380	This study
Ophiodaphne scripta	F	*Astriclypeus manni*	Koajiro, Kanagawa	*O. scripta*_KoajiroF	LC091381	This study
Ophiodaphne scripta	M	*Astriclypeus manni*	Koajiro, Kanagawa	*O. scripta*_KoajiroM	LC091382	This study
Ophiodaphne spinosa	F	*Clypeaster japonicus*	Tateyama, Chiba; -11.5M	*O. spinosa*_Tateyama1F	LC091383	This study
Ophiodaphne spinosa	M	*Clypeaster japonicus*	Tateyama, Chiba; -11.5M	*O. spinosa*_Tateyama2M	LC091384	This study
Ophiodaphne spinosa	F	*Clypeaster japonicus*	Tateyama, Chiba; -7M	*O. spinosa*_Tateyama3F	LC091385	This study
Ophiodaphne spinosa	M	*Clypeaster japonicus*	Tateyama, Chiba; -7M	*O. spinosa*_Tateyama4M	LC091386	This study
Ophiodaphne spinosa	F	*Clypeaster japonicus*	Iwachi, Shizuoka	*O. spinosa*_IwachiF	LC091387	This study
Ophiodaphne spinosa	M	*Clypeaster japonicus*	Iwachi, Shizuoka	*O. spinosa*_IwachiM	LC091388	This study
Ophiodaphne spinosa	F	*Clypeaster japonicus*	Ostuki, Kochi	*O. spinosa*_OstukiF	LC091389	This study
Ophiodaphne spinosa	M	*Clypeaster japonicus*	Ostuki, Kochi	*O. spinosa*_OstukiM	LC091390	This study
Ophiodaphne spinosa	F	*Clypeaster japonicus*	Kaiyo, Tokushima	*O. spinosa*_KaiyoF	LC091391	This study
Ophiodaphne spinosa	M	*Clypeaster japonicus*	Kaiyo, Tokushima	*O. spinosa*_KaiyoM	LC091392	This study

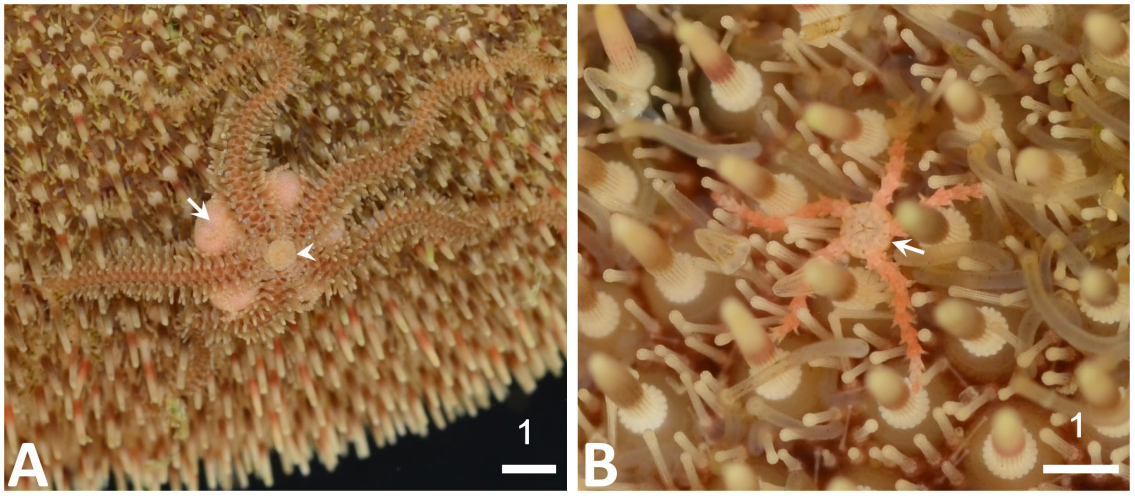


Fig. 2. Living, non-type specimens of *Ophiodaphne spinosa* sp. nov. **(A)** Female specimen (arrow) carrying a dwarf male (arrow head) on the ventral side of their host *Clypeaster japonicus* by hooking its dorsal arms to the spines of the host. Note the dwarf male with its ventral surface against that of the larger female, positioned mouth-to-mouth. **(B)** A solitary young male (arrow) on the ventral side of *C. japonicus*. Note it fixes its dorsal surface to the ventral surface of its host. Scale bars in millimeters.

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yses using MEGA ver. 5.2 were performed on the aligned DNA sequences of the partial COI gene. To select an appropriate nucleotide substitution model, jModelTest 0.1.1 (Posada, 2008) was used. MP trees were examined using the heuristic approach with 100 random initial trees. Statistical support for the ML and MP trees was evaluated by a nonparametric bootstrap test with 1000 resampling events.

RESULTS

Taxonomy

Ophiodaphne spinosa sp. nov.
(Figs. 2, 3)

Material examined. holotype; adult coupled female (NSMT E–7981; dd. 4.00 mm), collected by scuba diving, Tateyama Bay, Chiba Prefecture, Japan, 34°58′N, 139°47′E, 6 m, 13 August 2013. Paratypes; one adult coupled male (NSMT E–7982; dd. 1.04 mm) collected with holotype specimen, one young female (NSMT E–7983; dd 1.30 mm) and one juvenile (sex unknown, NSMT E–7984; dd 0.56 mm) collected by scuba diving, Otsuki, Kochi Prefecture, Japan, 32°47′N, 132°41′E, 16 m, 25 December, 2014.

Diagnosis. Dorsal surface of the disc in female covered by coarse scales. Radial shields contiguous. Radial shields and disc scales with transverse wavy grooves across the entire surface. Teeth pointed in female, 4–5 in a vertical row. Infradental papillae, oral papillae, adoral shields, and oral shield bearing numerous needle-like thorns. In life, disc and

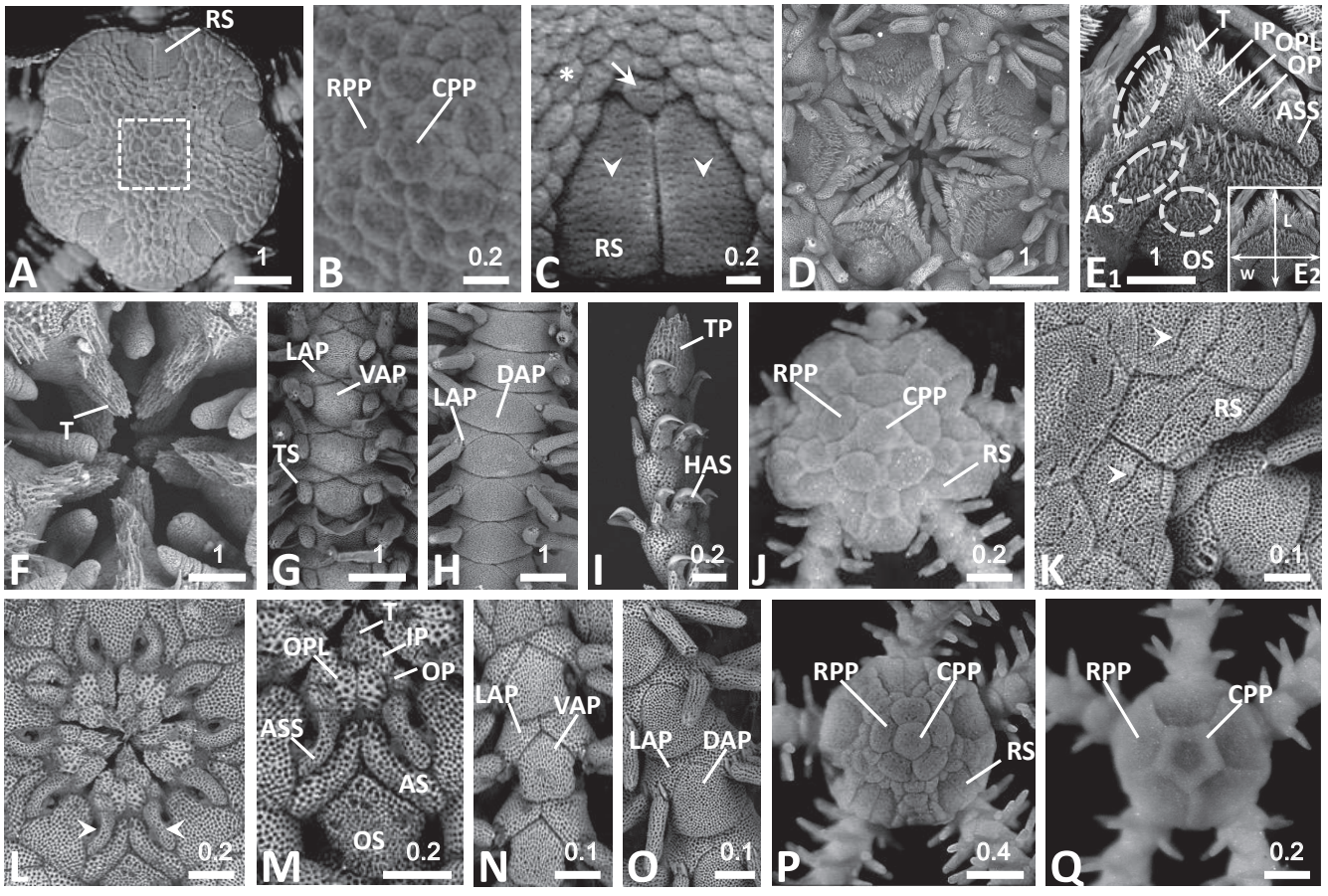


Fig. 3. Skeletal structures of *Ophoidaphne spinosa* sp. nov., holotype (pseudocopulated adult female, dd 4.00 mm: NSMT E–7981) **(A, B)**; paratype (pseudocopulated adult male, dd 1.04 mm: NSMT E–7982) **(J)**; paratype (young female, dd 1.30 mm: NSMT E–7983) **(P)**; paratype (juvenile, sex unknown, dd 0.56 mm: NSMT E–7984) **(Q)**; non-type specimen (female, dd 4.80 mm **(C–I)**, male, dd 0.80 mm **(K–O)**). **(D–I)** and **(K–O)** are SEM images. **(A)** Dorsal disc. Note contiguous radial shields (RS). **(B)** Enlargement of the square in **(A)**, note primary rosette (CPP: central primary plate, RPP: radial primary plate). **(C)** Enlargement of the radial shields. Note numerous wavy grooves (arrowheads) and a wedge-like disc scale (arrow) separating radial shields slightly. Many small disc scales with grooves (asterisk). **(D)** General overview of the oral frames. **(E)** Enlargement of one jaw angle. Components: tooth (T), an infradental papilla (IP), oral plate (OPL), oral papilla (OP), adoral shield spine (ASS), adoral shield (AS) and oral shield (OS). Note numerous thorns surrounded by dotted lines on the surfaces of an infradental papilla, oral papilla, adoral shields and oral shield **(E1)**. The measurement of the ratio of length (L) to width (W) of one jaw angle **(E2)**. **(F)** Mouth details. Tip of tooth (T), pointed. **(G)** Tentacle scale (TS), lateral arm plate (LAP) and ventral arm plate (VAP) of the ventral arm. Note ventral arm plates are pentagonal and in contact. **(H)** Dorsal arm. Note dorsal arm plates (DAP) are in contact. **(I)** Hook-shaped arm spines (HAS), distal arm tips. Note they are directed dorsally. TP, terminal plate. **(J)** Dorsal disc. **(K)** Grooves (arrowheads) on the radial shields and disc scales. **(L)** General overview of the oral frames. Note extremely curved adoral shield spines (arrowheads). **(M)** Enlargement of one jaw angle. **(N)** Ventral arm. Note ventral arm plates are pentagonal and not in contact. **(O)** Dorsal arm. Note dorsal arm plates are not in contact. **(P)** Dorsal disc. Note a few disc scales situated between radial shields and radial primary plate. **(Q)** Dorsal disc, formed by central primary plates and radial primary plates. Radial shields are rudimentary. Scale bars in millimeters.

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arms dark brown. Arms occasionally orange in males. Conspicuously dimorphic, male dwarfed.

Description. *Female*: Dorsal disc of the adult individual with one central primary plate, five radial primary plates, and five pairs of radial shields, surrounded by minute, imbricating disc scales (Fig. 3A, B). Maximum length of the radial shield (in a proximal-distal direction) one-fifth of the disc diameter. Radial shields completely contiguous along almost their entire length, slightly separated by a small wedge-like disc scale at the proximal end (Fig. 3C). The ventral interradii covered with uniform scales smaller than those of the dorsal side. Wavy transverse grooves visible on the surface of radial shields and disc scales (Fig. 3C). A pair of slender genital slits about 1 mm long present adjacent to the proximal arm. Each jaw consisting of pointed teeth, two triangular-shaped oral plates, infradental papillae, oral papillae at the outer edge of oral plates in addition to two trapezoid adoral shields and one rhomboid oral shield (Fig. 3D, E_1). Adoral shield spines at the second tentacle pore sit at the edge of the adoral shield, but do not cover the jaw, extending from the first ventral arm plate to the lateral oral papillae; distal oral papillae wider than high (Fig. $3E_1$). Adoral shields and oral shields conspicuously sunken longitudinally and transversally along the midline. Infradental and oral papillae sitting along the oral plate but articulated. Numerous needle-like thorns visible on the surface of infradental papillae, oral papillae, adoral shields, and oral shield (Fig. $3E_1$). Ratio of length-to-width of one jaw angle approximately 1.12 (Fig. $3E_2$). Teeth pointed, spine like (Fig. 3F). Ventral arm plates pentagonal with convex distal edge, obtuse proximal angle and weakly concave lateral edges (Fig. 3G). Dorsal arm plates wide bell-shaped with convex proximal and distal edges, about twice as wide as long (Fig. 3H). Ventral and dorsal arm plates abutted by lateral arm plates and in contact (Fig. 3G, H). An ovoid tentacle scale articulated on the lateral arm plate and covering the tentacle pore (0.1 mm in diameter) completely (Fig. 3G). Ratio of length of the longest arm to the disc diameter approximately 3.4. Six to seven cylindrical arm spines formed on the basal arm joints, situated on both sides of the lateral arm plates. Middle arm spine (0.6 mm long), longer than the ventralmost and dorsalmost arm spines. Arm spines toward distal end of the arm, of which three transformed into curved, glassy hook-shaped arm spines. Last several arm segments bearing hook-shaped arm spines, the tips directed dorsally (Fig. 3I). In life, the disc and arms dark brown in color.

Male: Dorsal disc of the adult individual with one central primary plate, five radial primary plates, and five pairs of radial shields, covered by two small disc scales between radial primary plates and two larger round ones beyond radial primary plates to disc edge. There also two narrow ones at either side of the distalmost scale (Fig. 3J). Maximum length of the radial shield in a proximal-distal direction approximately one-fifth to one-quarter of the disc diameter. Radial shields completely contiguous along their whole length and wedge-like disc scale absent (Fig. 3J, K). Wavy transverse grooves visible on the surface of radial shields and disc scales (Fig. 3K). A pair of slender genital slits about 0.2 mm long, adjacent to the proximal arm. Jaws partly covered by curved adoral shield spines at the second tentacle pore, extending from the first ventral arm plate to the mouth angle (Fig. 3L). Each jaw consisting of tooth, two oral plates, infradental papillae and oral papillae at the outer edge of oral plates accompanied with two trapezoid adoral shields and one rhomboid oral shield (Fig. 3M). A few rudimentary thorns on the surface of infradental and oral papillae visible (Fig. 3M). Three short cylindrical arm spines (0.2 mm long) formed on the basal arm joints, situated on both sides of the lateral arm plates. Arm spines transformed into glassy hook-shaped arm spines in distal arm segments, the tips directed dorsally. Pentagonal dorsal and ventral arm plates abutted by lateral arm plates and not in contact (Fig. 3N, O). Tentacle scales lacking on all arm joints (Fig. 3N). Ratio of length of the longest arm to the disc diameter approximately 3.4. In life, the disc and arms dark brown in color, with the latter occasionally orange.

Young and juvenile: Disc of the young female round, not pentagonal, with one central primary plate, five radial primary plates, five pairs of radial shields, and minute disc scales (Fig. 3P). Thorns visible along the outer edges of oral plates similar to those of adult females. Disc of the juvenile round with one central primary plate, five radial primary plates, and rudimentary radial shields (Fig. 3Q). During ontogenesis in males, the disc changing from completely

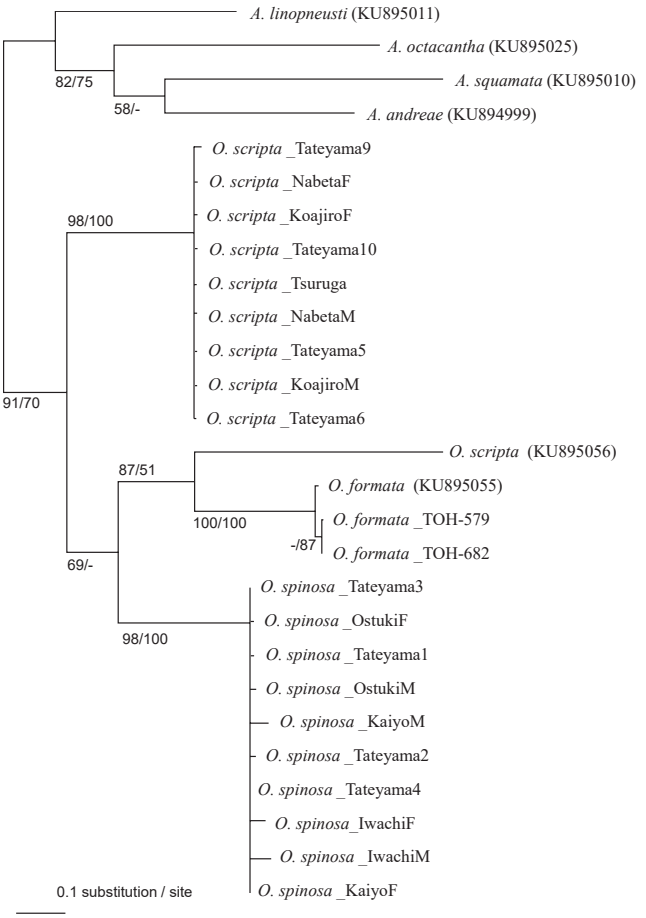


Fig. 4. Maximum likelihood (ML) tree of amphiurid species based on partial cytochrome oxidase subunit I (COI) gene sequences. The GTR+I+G model was used for the analysis. Bootstrap probabilities larger than 50% for the ML and maximum-parsimony (MP) analyses are noted.

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round to round with deep incisions at the arms in outline with growth, and the surface of the dorsal disc gradually covered by radial shields and small disc scales.

Etymology. The species name is the Latin adjective, *spinosa*, referring to the numerous thorns on jaw ossicles in females.

Comparative morphological observations

A comparison of the morphological characteristics of four species of *Ophiodaphne* is presented in Table 1. In females, the radial shields on the surface of the disc in *O. spinosa* sp. nov., *O. scripta* (non-type specimens collected in Japan), and *O. materna* (syntypes) were contiguous (Figs. 3A, C, 5A, B, K, L), but were divergent in *O. formata* (holotype) (Fig. 5P, Q). Wavy grooves were formed across the entire surface of the radial shields in both sexes of *O. spinosa* sp. nov. (Fig. 3C). Transverse parallel grooves on *O. scripta* females were situated along the inner edge of the radial shields (Fig. 5B), but these were rudimentary in males. Grooves were absent on *O. materna* and *O. formata* (Fig. 5K, L, P, Q). Jaw ossicles in *O. scripta* females did not possess thorns, which were limited to females of *O. spinosa* sp. nov. (Figs. 3D, E_1, 5D), but *O. scripta* females had serrated fringes along the outer edge of the oral plates (Fig. 5C). The shape of the tip of the tooth was pointed in *O. spinosa* sp. nov., *O. materna* and *O. formata* (Figs. 3F, 5M, R), but wide and blunt in *O. scripta* (Fig. 5E). The tentacle scale of *O. scripta* is unusually attached to the lateral arm plate (Fig. 5F), while in *O. spinosa* sp. nov., *O. materna*, and *O. formata* it sits at the side of the ventral arm plate (Figs. 3G, 5N, S). Furthermore, in *O. scripta* dorsal arm plates are very broad and bell-shaped with extensions of the lateral arm plates

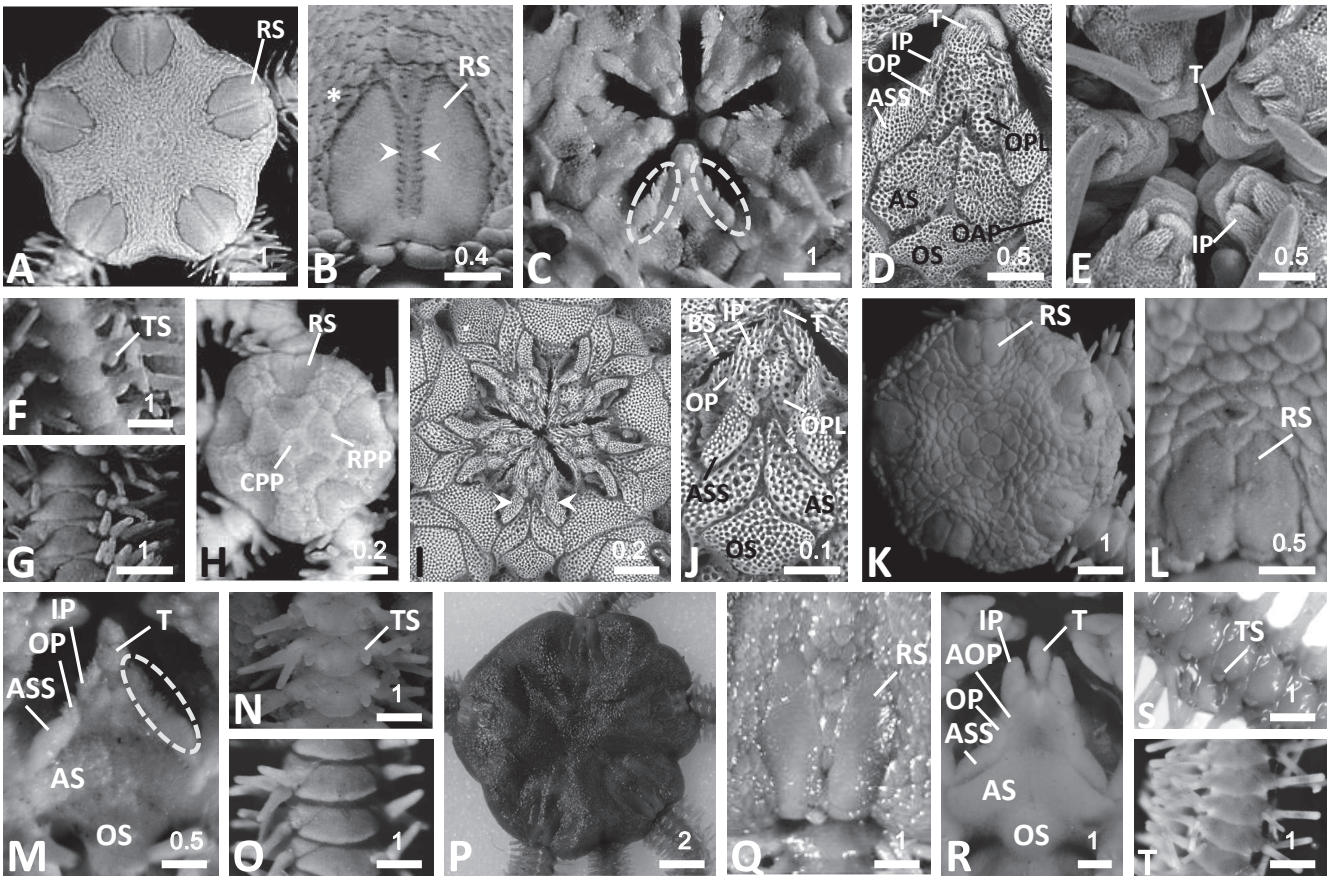


Fig. 5. Skeletal structures of *Ophiodaphne scripta*, *O. materna* and *O. formata*. **(D, E, I, J)** are SEM images. *Ophiodaphne scripta* collected in Japan (non-type specimens: **(A–J)**, pseudocopulated adult female dd 4.10 mm: **(A)**, pseudocopulated adult male dd 1.04 mm: **(H)**). *Ophiodaphne materna* (syntype: pseudocopulated adult female dd 4.20 mm: OPH–16) **(K–O)**. *Ophiodaphne formata* (holotype: adult female dd 12.5 mm: ZMA. V. ECH. O. 2006, **(P)** courtesy of Ms. J. Bleeker) **(P–T)**. **(A)** Dorsal disc. Note relatively large radial shields (RS) and small disc scales. **(B)** Enlargement of the radial shields. Note contiguous radial shields with grooves along inner edge (arrowheads) and many small disc scales with grooves (asterisk). **(C)** General overview of the oral frames. Note papillae with fringed edges surrounded by dotted lines. **(D)** Enlargement of one jaw angle. Note three papillae (IP, OP and ASS). **(E)** Mouth details. Tip of tooth (T), wide and blunt. **(F)** Ventral arm with tentacle scales. **(G)** Dorsal arm. **(H)** Dorsal disc. **(I)** General overview of the oral frames. Note extremely curved adoral shields spines (arrowheads). **(J)** Enlargement of one jaw angle. BS, buccal scale. **(K)** Dorsal disc. **(L)** Enlargement of the radial shields. Note contiguous radial shields and disc scales with no grooves. **(M)** Enlargement of one jaw angle. Note three papillae (IP, OP and ASS) with serrated fringed edges surrounded by dotted lines. **(N)** Ventral arm with tentacle scales. **(O)** Dorsal arm. **(P)** Dorsal disc. **(Q)** Enlargement of the radial shields. Note divergent radial shields. No grooves on the radial shields and disc scales. **(R)** Enlargement of one jaw angle. Note pointed tooth and four papillae (IP, AOP, OP and ASS) without thorns. AOP, additional small oral papilla. (S) Ventral arm with tentacle scales. **(T)** Dorsal arm. Abbreviations as in Fig. 3. Scale bars in millimeters.

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separating each one (Fig. 5G), while in three species mentioned above the dorsal arm plates are in contact (Figs. 3H, 5O, T). The dorsal disc of the adult male of *O. scripta* is similar to that of *O. spinosa* sp. nov. (Fig. 5H). The adoral shield spines in male *O. spinosa* sp. nov. and *O. scripta* were long and curved, differing from those of females (Figs. 3L, M, 5I, J). The ratio of length to width of one jaw angle in female specimens from Japan was significantly smaller in *O. spinosa* sp. nov. (non-type specimens) than in *O. scripta* (non-type specimens) (*t* test for both species, $P < 0.001$, Table 1), but no differences were found between males of the two species. Adoral shields and oral shield were distinctly sunken along the midline in females of both species. The numbers of arm spines on the basal arm joints was six to seven in females and three to four in males of *O. spinosa* sp. nov., compared to four to five in females and three in males of *O. scripta*. On *O. spinosa* sp. nov. and *O. scripta* females collected in Tateyama Bay, the numbers of disc scales on the dorsal disc were 667 ± 151 ($n = 26$; adults, dd 3.61–5.32 mm) and 1444 ± 205 ($n = 8$; adults, dd 4.23–5.55 mm), respectively. The values of the former were significantly less than those of the latter (*t* test, $P < 0.001$). There were no distinctions in the external morphological characteristics on males of the two species except for the grooves. The color of the disc and arms in life is a darker brown in *O. spinosa* sp. nov. than in *O. scripta*.

Molecular analyses

We obtained a total of 10 haplotypes from the partial COI genes of ten *O. spinosa* sp. nov. specimens from four collection sites (Accession Numbers LC091383–LC091392, Table 2) and in total four haplotypes from the partial COI genes of nine specimens of Japanese *O. scripta* from four collection sites (Accession Numbers LC091374–LC091382, Table 2). We analyzed a total of 26 haplotypes from 27 specimens of seven amphiurid species. The aligned amino acid sequences of seven species from two genera contained no gaps (insertion/deletion) and we confined our analyses to a specific subset of 617 bp of the partial COI sequences. The average base frequencies of all three codon positions from the 26 haplotypes of amphiurid specimens were 25.6% A, 25.9% C, 18.8% G, and 29.7% T, with an A+T composition frequency of 55.3%. Among the three *Ophiodaphne* species, sequence differences within species were lower (0%–1.1%, 0–7/617 bp in Japanese *O. scripta*; 0.2%–1.1%, 1–7/617 bp in *O. formata*; 0.2%–3.1%, 1–19/617 bp in *O. spinosa* sp. nov.) than sequence differences among the three species of *Ophiodaphne* (15.2%–19.3%, 94–119/617 bp). DNA sequence of Japanese *O. scripta* and *O. scripta* (KU895056) formed different node. Furthermore, sequence differences between *O. scripta* (KU895056) and *O. formata* was high (16.5%–16.9%, 102–104/617 bp). The mean sequence divergence among the seven species of Amphiuridae was 17.7%.

For data with all codon positions, the general time-reversible model with invariant rates among sites and gamma distribution (GTR+I+G) was selected as the appropriate model for phylogenetic analyses of the partial COI gene, according to Akaike's information criterion (AIC). Figure 4 shows the ML tree using the GTR+I+G substitution model. As the topologies of the ML and MP phylogenetic trees were nearly identical, the strict-consensus MP tree was not shown. The monophyly of each *Ophiodaphne* species was supported by high bootstrap values, and the monophyly of the genus *Ophiodaphne* was supported by relatively high bootstrap values (ML = 91%, MP = 70%).

DISCUSSION

In *Ophiodaphne*, morphologically detailed examinations of the type specimen of *Ophiodaphne scripta* showed strong similarities with specimens associated with *Astriclypeus manni* collected from Japan (Table 1), leading to their identification as *O. scripta* (Koehler, 1904). *Ophiodaphne spinosa* sp. nov. clearly differs from *O. scripta* in the smaller number of disc scales on the dorsal disc surface, the many wavy grooves on radial shields and disc scales, the presence of numerous thorns on the jaw ossicles, the pointed teeth, the smaller ratio of length to width of one jaw angle, and the number of arm spines, while the larval developmental mode of the two species, ophiopluteus larvae, are very similar (Tominaga et al., unpublished). Specimens of *O. scripta* (Parameswaran et al., 2013) and *O. materna* (Devaney, 1974) seem quite likely to be *O. spinosa* sp. nov. based on our assessment of the following characteristics; wavy grooves, thorns, and teeth.

Females of the three *Ophiodaphne* species, *O. spinosa* sp. nov., *O. scripta*, and *O. materna* have approximately the same range of disc diameters, but their disc diameters are smaller than those of *O. formata*. No individuals larger than 7 mm dd in adults of these three species were observed. In *O. scripta*, females with a dd larger than 3.7 mm had ripe gonads and spawned (Tominaga and Komatsu, 2013). Females of *O. spinosa* sp. nov. with a dd of at least 2.0 mm were mature (Tominaga, unpubl.). Therefore, *O. formata* seems to be a larger ophiuroid than the other *Ophiodaphne* congeners.

Radial shields of adult *O. spinosa* sp. nov., *O. scripta*, and *O. materna* are contiguous. The radial shields of young individuals of *O. spinosa* sp. nov. and *O. scripta* are also contiguous. Koehler (1905) described the radial shields of *O. formata* as divergent based on the type specimen collected from Sumbawa Island, Indonesia. In addition, individuals of adult *O. formata* (dd ≥ 9.0 mm) collected from the Philippines, Western Australia, and New Caledonia also have divergent radial shields (Guille, 1981; Guille and Vadon, 1986; Ratnasingham and Hebert, 2007; O'Hara, Victoria Museum, pers. comm.), while a young female with a dd of 1.3 mm had contiguous radial shields (Guille, 1981). The radial shields of *O. formata* may become divergent as it grows. Thus, *O. formata* can be distinguished from *O. spinosa* sp. nov., *O. scripta*, and *O. materna*, all of which have contiguous radial shields.

The presence or absence of grooves on the radial shield in females has been considered an important taxonomic characteristic (Clark, 1967). There are three types of radial shield grooving in *Ophiodaphne* congeners. First, grooves are not formed, as in *O. materna* syntypes and *O. formata* holotype. Second, grooves are formed at the juncture of the radial shields, as in *O. scripta* syntype and other specimens from Madagascar (Cherbonnier and Guille, 1978). Most Japanese *O. scripta* specimens also have such grooves. Third, wavy grooves over the whole surface of the radial

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shields are formed as in *O. spinosa* sp. nov.

The shape of a jaw angle in females, *O. spinosa* sp. nov. is characterized by thorny jaw ossicles. Females of *O. scripta*, *O. materna*, and *O. formata* have no thorns on the jaw ossicles. The habitats of *O. spinosa* sp. nov. and their hosts *C. japonicus* are coarser seafloors of mixed sand and pebble grains. Therefore, *O. spinosa* sp. nov. may have developed thorns on the papillae and jaw ossicles to scrape organic material off large grain surfaces. In contrast, *O. scripta* and its host *A. manni* live on sandy benthic substrates. Perhaps because its diet consists of fine sandy grains, *O. scripta* lacks thorns, which may be a trait that is adaptive to the living environment of commensal ophiuroids and their hosts.

With regard to the arrangement of the papillae at the outer edge of the jaw ossicle, *O. spinosa* sp. nov., *O. scripta*, and *O. materna* specimens have three papillae (an infradental papilla, an oral papilla, and an adoral shield spine). As noted by Clark (1967), *O. scripta* is characterized by large distal papillae forming a continuous fringe. The holotype of *O. formata* has four papillae (an infradental papilla, an additional small oral papilla, an oral papilla, and an adoral shield spine). Guille (1981) also described four papillae in *O. formata*. There seems to be morphological diversification in shape and number of papillae in females of *Ophiodaphne* congeners, which may be related to feeding behavior.

DNA sequences can provide useful nametags for species identification if they show species-specific variation. Recently, Hugall et al. (2016) developed an exon-capture system for phylogenetic analysis using next-generation sequencing. However, a partial mitochondrial COI gene region (“Folmer” region) has been emerging as a standard barcoding region (Ratnasingham and Herbert, 2007), and BOLD supports application of DNA barcode data. In this study, the intraspecific variations within each of the three species of *Ophiodaphne* (0–3.1%) were much smaller than the interspecific variations among them (15.2–19.3%). These results suggest that COI sequences can be useful as DNA barcodes for distinguishing morphologically similar species of *Ophiodaphne*. Monophyly of each species was supported by high bootstrap values, but monophyly of a group of *O. scripta* (KU895056), *O. formata*, *O. spinosa* sp. nov. was supported by low bootstrap values. Monophyly of an inner group of *O. scripta* (KU895056) and *O. formata* was also supported by low bootstrap values. There may be two cryptic species within *O. scripta*. Thus, the details of *Ophiodaphne* phylogeny remain unclear.

There are variations in the morphological characteristics among the four *Ophiodaphne* congeners, *O. spinosa* sp. nov., *O. scripta*, *O. materna*, and *O. formata*. In particular, *O. materna* is not considered to be a synonym of *O. formata* based on some morphological characteristics; the disc diameter, the degree of contiguity of the radial shield and the shape of the jaw angle. Given these phylogenetic results, four *Ophiodaphne* species are distinguishable. To examine the classification of the characteristic *Ophiodaphne* species further, additional morphological observations, molecular analyses, and information of ophiuroid–host commensalism, in particular *O. materna* and *O. impellera*, will be required in future studies.

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COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

HT designed this study. HT and MH prepared figures and tables, and wrote the manuscript. MH performed DNA extraction, PCR amplification, sequencing, and phylogenetic analysis. HI, M Kiyomoto, and M Komatsu collected specimens with HT and supervised this study.

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