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

Hideyuki Tominaga1*, Mamiko Hirose2†, Hikaru Igarashi3, Masato Kiyomoto2, and Miéko Komatsu4

1-24-9 Hoei, Fukui 910-0004, Japan
2Marine and Coastal Research Center, Ochanomizu University, Chiba 294-0301, Japan
3Department of Biology, Faculty of Science, University of Toyama, Toyama 930-8555, Japan
4University of Toyama, Toyama 930-8555, Japan

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 (Koeehler, 1904), Ophiodaphne formata (Koeehler, 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...
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). 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,
A new species of brittle star

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

The type species, 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 (NBC) 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 marginal disc shield, groove (G), as the distance from the base of the arm to the opposite marginal disc shield, arm length (AL), as the distance from the base of the arm to the opposite marginal disc shield.

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 AstercoCOI (TTCWCAAACWCTAGATATW-GGAAC) (designed in this study) and COIcER (TCTGTTGCTTAC-GTCCATTCCTACTGTGAACTRTTG) (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 Amphiriidae (Amphioplus andreae KU894999, Amphipholis linopneusti KU895011, Amphipholis squamata KU895010; Amphipholis 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) analyses were performed using PAUP* (Swofford, 2002). The maximum-likelihood (ML) analysis was performed using the GTR+I+G model (models were selected using the Akaike information criterion (AIC) implemented in jModelTest 2.1.5 [Darriba et al., 2012]). The matrix of pairwise base differences was 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) analysis were performed.

Table 1. Comparison of the morphological characteristics of four congeners of Ophiodaphne species. aValues in mm. Asterisks mean average. bGrooves on the radial shields and dorsal disc scales. cThorns on the jaw ossicles. dRatio of length to width of one jaw angle. NA; no data available.

<table>
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<tr>
<th>Species</th>
<th>Locality</th>
<th>Disc diameter</th>
<th>Radial shields</th>
<th>Groove</th>
<th>Thorns</th>
<th>Teeth</th>
<th>Ratio</th>
<th>Arm spines</th>
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<td>(Koehler, 1904)</td>
<td>Oman</td>
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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.

<table>
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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.
RESULTS

Taxonomy

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

Material examined. holotype; adult coupled female

(yes 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.

Fig. 3. Skeletal structures of Ophiodaphne 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.

(yes 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

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. Infracental papillae, oral papillae, adoral shields, and oral shield bearing numerous needle-like thorns. In life, disc and...
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). 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). 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). Ratio of length-to-width of one jaw angle approximately 1.12 (Fig. 3E). 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 dorsal 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

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**Fig. 4.** Maximum likelihood (ML) tree of amphipod 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.
A new species of brittle star, **Ophiodaphne scripta** 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, E1, 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.
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 amphurid species. The aligned amino acid sequences of seven species from two genera contain 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 amphurid 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 (Koechler, 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, ophiuroid 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. Koechler (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, 1987). There are three types of radial shield growing 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 *Ophiiodaphne* 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 *Ophiiodaphne* (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 *Ophiiodaphne*. 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 *Ophiiodaphne* phylogeny remain unclear.

There are variations in the morphological characteristics among the four *Ophiiodaphne* 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 *Ophiiodaphne* species are distinguishable. To examine the classification of the characteristic *Ophiiodaphne* 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 Kyomoto, and M Komatsu collected specimens with HT and supervised this study.

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