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Molecular evidence for synonymy of the genera *Moroteuthis* and *Onykia* and identification of their paralarvae from northern Hawaiian waters

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It has been claimed that most squid species in the genus *Onykia* may be immature stages of species in the genus *Moroteuthis*. To evaluate the generic status of *Moroteuthis* and *Onykia* and to identify paralarvae collected in northern Hawaiian waters, we performed morphological investigation and nucleotide sequence analysis of the mitochondrial cytochrome oxidase I (COI) gene. Of 42 *Onykia* paralarvae (1.8–8.5 mm dorsal mantle length, DML) examined, 41 had a nucleotide sequence identical to that of *M. robusta* and one (designated *Onykia* sp. A) could not be assigned to any known *Moroteuthis* species. Nucleotide sequence diversity estimates based on Kimura's two-parameter distances between *Onykia* sp. A and *Moroteuthis* spp. (0.109–0.150) fell well within the range of congeneric species, suggesting that *Onykia* sp. A is a member of the genus *Moroteuthis*. Molecular data supported the conclusion that the genus *Moroteuthis* is a junior synonym of the genus *Onykia*. Morphological investigation revealed that paralarvae of *O. robusta* (= *M. robusta*) <4.0 mm DML were distinct from other *Onykia* paralarvae in the chromatophore pattern on the mantle. The key characters for distinguishing *O. robusta* paralarvae from *Onykia* sp. A were the number and arrangement of chromatophores on the funnel.

INTRODUCTION

The squid family Onychoteuthidae presently comprises five genera (*Onychoteuthis*, *Ancistroteuthis*, *Onykia*, *Moroteuthis*, and *Kondakovia*; Kubodera et al., 1998), however, the taxonomic status of some genera is controversial. Neither paralarvae nor juveniles of the genus *Moroteuthis* have been described, and only one adult form of the genus *Onykia* (*O. rancureli*) has been reported (Kubodera et al., 1998). It has been suggested that some *Onykia* species are actually juvenile stages of *Moroteuthis* species (Tsuchiya & Okutani, 1991).

Paralarvae of *Onykia* species are distributed in surface waters and are frequently caught by plankton nets. Although paralarvae of this genus have been described (Pfeffer, 1884, 1900, 1912; Okutani, 1968), species identification has not been established. Most oceanic squids have few chromatophores on the body during the paralarval stages, whereas *Onykia* paralarvae are characterized by numerous chromatophores on the mantle, head and arms (Clarke, 1992). The arrangement of chromatophores is often useful in identifying species at the paralarval stage (Okutani & McGowan, 1969; Young & Harman, 1987; Wakabayashi et al., 2002). However, the large number of chromatophores characteristic of *Onykia* paralarvae may hinder species identification within this genus. To date, most paralarvae of *Onykia* species have been identified to only a single species, *O. carriboea* Lesueur, 1821, based on this character, but it is likely that *O. carriboea* is a species complex considering the number of adult species that are. Molecular genetic analyses may resolve the problems mentioned above, and restriction fragment length polymorphism (RFLP) and/or direct nucleotide sequencing analyses based on the polymerase chain reaction (PCR) have been used to successfully analyse ommastrephid squid paralarvae collected in northern Hawaiian waters (Wakabayashi et al., 2006).

In this study, we present molecular evidence for synonymy of the squid genera *Moroteuthis* and *Onykia* and report results of morphological and molecular genetic analyses to identify species of *Onykia* collected from northern Hawaiian waters. We also briefly discuss the *Onykia* life cycle, which has not been previously described.

MATERIALS AND METHODS

Squid samples

Standard specimen

Adult samples from six onychoteuthid squids were used as standards (Table 1). *Moroteuthis robusta* was obtained from the stomach content of a sperm whale caught off Sanriku, Japan, and *M. loennbergii* from a bottom trawl off Sanriku, Japan. *Moroteuthis robsoni* and *M. ingens* distributed in the southern hemisphere were obtained from squid jigging in New Zealand waters. Two species of the genus *Onychoteuthis* (*O. banksii* and *O. borealijaponica*) were collected by mid-water trawl off Sanriku. The nucleotide sequences for *M. robusta*, *M. knipovitchi*, *Onychoteuthis compacta* and *Watasenia scintillans* (family Enoploteuthidae) were derived from Anderson (2000), Lindgren et al. (2004), Carlini & Graves (1999) and Yokobori et al. (2004), respectively.

Table 1. *Sampling localities of adult Onychoteuthid samples used in this study.*

Species	Locality	N	Vessel
Moroteuthis robusta	Off Sanriku, Japan	1	'Nisshin-Maru'
M. loennbergii	Off Sanriku, Japan	1	'Wakataka-Maru'
M. robsoni	New Zealand	1	–
M. ingens	New Zealand	1	'Hakurei-Maru'
Onychoteuthis banksii	Off Sanriku, Japan	1	'Marutei-Maru'
O. borealijaponica	Off Sanriku, Japan	1	'Marutei-Maru'

Paralarvae

Onykia paralarvae were collected north of the Hawaiian Archipelago (26°30'–37°N 155°–162°30'W; Figure 1) in 2002 and 2003 by the National Research Institute of Far Seas Fisheries aboard the RV 'Shunyo-Maru'. In total, 42 paralarvae were collected using a large larva net (2 m mouth diameter; 526-μm mesh) in surface horizontal tows at ship speeds of 1.5 to 2 knots. The sorted paralarval samples were frozen or preserved in 70% ethanol on-board, transferred to the laboratory, and identified based on morphology. All paralarval samples were deposited at the National Science Museum in Tokyo, Japan.

Crude DNA was extracted from the mantle of each standard sample and one of the fins of each paralarva using a DNA extraction kit (GenomicPrep Cells and Tissue DNA Isolation Kit; Amersham Biosciences, Piscataway, NJ, USA).

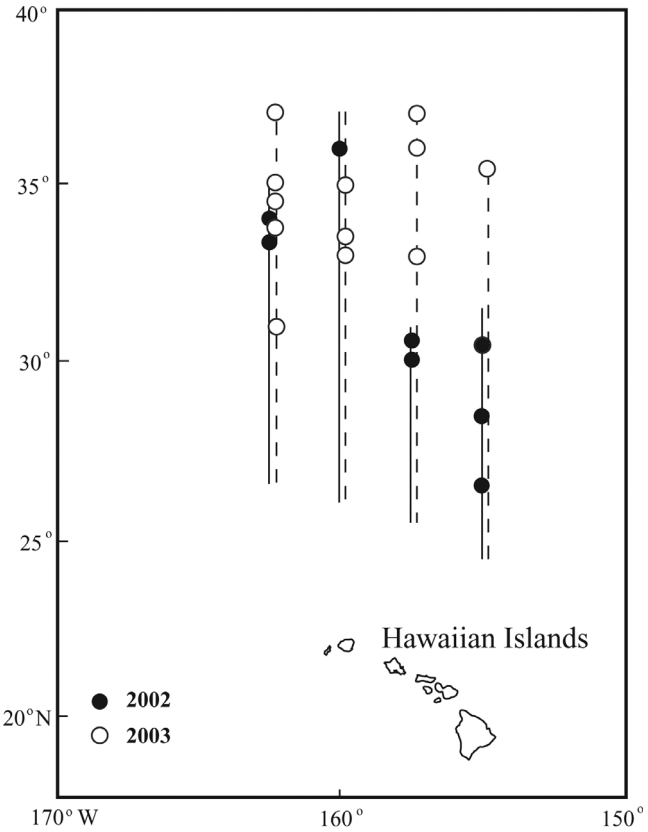

Figure 1. Map of the sampling area showing transects surveyed during 2002 (solid line) and 2003 (dashed line). Closed and open circles indicate stations where paralarvae of *Onykia* were collected in 2002 and 2003, respectively.

PCR amplification

We used primers LCO1490 and HCO2198 (Folmer et al., 1994) for standard samples and primers LCO1495N (5'-ACAAAYCATAAAGAYATTGG-3') and COI6785R1 (5'-GATAATATATGRTGGGCTCA-3') for paralarval samples to amplify a partial segment of the mitochondrial cytochrome oxidase I (COI) gene.

Polymerase chain reaction amplification was carried out in 10 μl reaction mixtures containing 5 μl of 2×GC buffer, 1 mM of each dNTP, 0.4 μM of each primer, 0.4 units of LA Taq polymerase (TaKaRa, Shiga, Japan), and the DNA template. The reaction mixtures were preheated at 95°C for 3 min, followed by 30 amplification cycles (95°C for 40 s, 46°C for 40 s, and 72°C for 1.5 min), with a final extension at 72°C for 5 min.

Nucleotide sequence and RFLP analyses

Nucleotide sequence analysis was performed on all standard samples and on five paralarval samples (designated *Onykia* sp. 16-1, 18-1, 22-1, 46-4 and 50-1) having different dorsal mantle lengths (DML; 2.0, 4.0, 6.3, 7.5, and 8.5 mm). The PCR products were treated with ExoSAP-IT (Amersham Biosciences) to remove the oligonucleotide primers. Sequences were generated on an automated sequencer (ABI Prism310; Applied Biosystems, Foster City, CA, USA) using the ABI Big-dye Ready Reaction kit (Applied Biosystems) following the standard cycle sequencing protocol. The nucleotide sequences obtained using both forward and reverse primers were edited and aligned on GENETYX v. 6.1 (GENETYX, Tokyo, Japan), considering their deduced amino acid sequences, and then imported into MEGA v. 3.1 (Kumar et al., 2004) to calculate sequence divergence (Kimura's two-parameter distance: K2P) and to construct a neighbour-joining (NJ) phylogenetic tree.

According to the nucleotide sequence data obtained, we selected an endonuclease (*Fok* I) for species identification using RFLP. The PCR products were directly digested by this enzyme and electrophoresed through a 2.5% agarose gel (Biogel, BIO101; Regent Medical, Manchester, UK) for 2 h, stained with ethidium bromide, and photographed.

The nucleotide sequences reported here were deposited in DDBJ/EMBL/GenBank under accession numbers AB264116–AB264122.

Morphological observations

A binocular microscope with a micrometer was used to observe and measure the paralarvae. The measurements, indices, and terminologies used here follow Roper & Voss (1983). The specimens used for scanning electron microscope (SEM) observations were dehydrated in a graded series of ethanol and t-butyl alcohol, dried using a freeze dryer (ES-2030; Hitachi, Ibaraki, Japan), and coated with gold. A SEM (SM-200; Topcon, Tokyo, Japan) was used to observe the tentacles, arms and mouthparts.

Table 2. *Estimates of mean nucleotide sequence diversity based on Kimura's two-parameter distance between species.*

	M.rst	M.lbg	M.rsn	M.igs	M.kpv	Ok50-1	O.com	O.bks	O.bjp
M.rst									
M.lbg	0.109								
M.rsn	0.106	0.125							
M.igs	0.142	0.147	0.140						
M.kpv	0.176	0.167	0.205	0.124					
Ok50-1	0.120	0.109	0.124	0.138	0.150				
O.com	0.194	0.199	0.206	0.157	0.160	0.204			
O.bks	0.180	0.199	0.218	0.145	0.160	0.206	0.103		
O.bjp	0.193	0.197	0.232	0.164	0.159	0.215	0.138	0.102	
Ws	0.224	0.229	0.257	0.192	0.217	0.231	0.225	0.221	0.214

M.rst, *Moroteuthis robusta*; M.lbg, *M. loennbergii*; M.rsn, *M. robsoni*; M.kpv, *M knipovitch*; Ok50-1, *Onykia* sp. 50-1; O.com, *Onychoteuthis compacta*; O.bks, *O. banksii*; O.bjp, *O. borealijaponica*; Ws, *Watasenia scintillans*.

RESULTS

Nucleotide sequence analysis

No insertions or deletions were observed in the aligned 554-bp fragments, and 186 variable sites were observed among sequences of ten standard samples and five paralarvae. The nucleotide sequence diversity estimates (π) based on K2P distances between species are shown in Table 2. No substitutions were observed among four paralarval specimens (*Onykia* sp. 16-1, 18-1, 22-1, 46-4). The nucleotide sequence diversity between these four *Onykia* sp. paralarvae and *Moroteuthis robusta* was very small (ranging from 0 to 0.013), and a phylogenetic tree showed that these paralarvae clearly grouped with *M. robusta* (Figure 2). One paralarva (*Onykia* sp. 50-1) was not assigned to any *Moroteuthis* species analysed. The sequence divergence (K2P) between *Onykia* sp. 50-1 and *Moroteuthis* species ranged from 0.109 to 0.15, and much larger divergence (0.204–0.215) was observed between *Onykia* sp. 50-1 and the *Onychoteuthis* species. The sequence divergence between species within a genus ranged from 0.102 to 0.205 in *Moroteuthis* and from 0.102 to 0.138 in *Onychoteuthis*. The sequence divergence between species from different families ranged from 0.192 (*M. ingens* vs *Watasenia scintillans*) to 0.257 (*M. robsoni* vs *W. scintillans*). These results indicate that *Onykia* sp. 50-1 is a paralarva of unknown species in the genus *Moroteuthis*, and it was re-designated *Onykia* sp. A.

Our NJ tree supported the monophyly of the genus *Onychoteuthis*, but not the monophyly of the genus *Moroteuthis* (Figure 2). *Moroteuthis knipovitchi* was observed to be more closely related to species of the genus *Onychoteuthis* than to *Moroteuthis*.

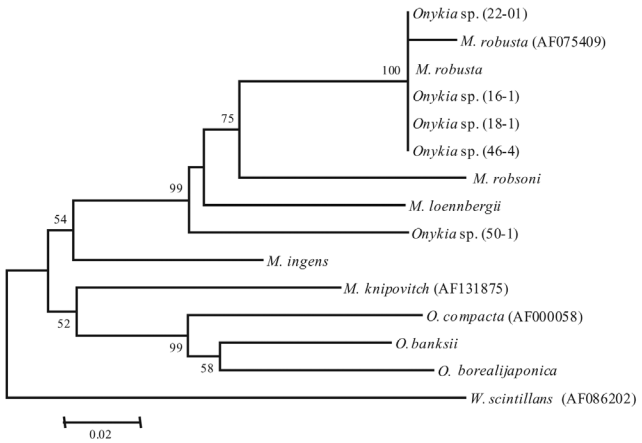

Figure 2. NJ tree of standard species and paralarvae. Bootstrap values higher than 50% are shown.

RFLP analysis and species identification of paralarvae

Since no notable morphological difference was observed between paralarvae of *M. robusta* (four *Onykia* sp.) and *Onykia* sp. A, we compared restriction profiles of all paralarval specimens with those of adult *M. robusta* and *M. loennbergii*. Distinct restriction profiles were observed between adult *M. robusta* and *M. loennbergii*, in which the estimated sizes of the restricted fragments were 677 and 177 bp (Figure 3, lane 2) in *M. robusta* and 359, 318, and 177 bp (Figure 3, lane 3) in *M. loennbergii*. All 41 paralarvae examined shared an identical restriction profile with the adult standards of *M. robusta*, while the restriction profile (495 and 359 bp) of *Onykia* sp. A (Figure 3, lane 4) was distinct from those of *M. robusta* and *M. loennbergii*.

Nomenclature

Tsuchiya & Okutani (1991) suggested the genus *Moroteuthis* Verrill, 1881 is a junior synonym of the genus *Onykia* Lesueur, 1821. Our sequence data support the view, and hereafter, we use the genus *Onykia* (Lesueur, 1821) for all species of the genus *Moroteuthis* (Verrill, 1881) except for '*Moroteuthis*' *knipovitchi*.

Descriptions of paralarvae

In total, 41 *O. robusta* paralarvae ranging from 1.8 to 8.5 mm DML and one *Onykia* sp. A paralarva of 7.5 mm DML were collected.

Onykia robusta *(Figures 4–6)*

The mantle is rather thin, dome-shaped, and ornamented by about 50 square-shaped chromatophores that are densely packed on each side of the dorsal and ventral mantle at 1.8 mm DML. At 4.0 mm DML, the mantle is muscular and

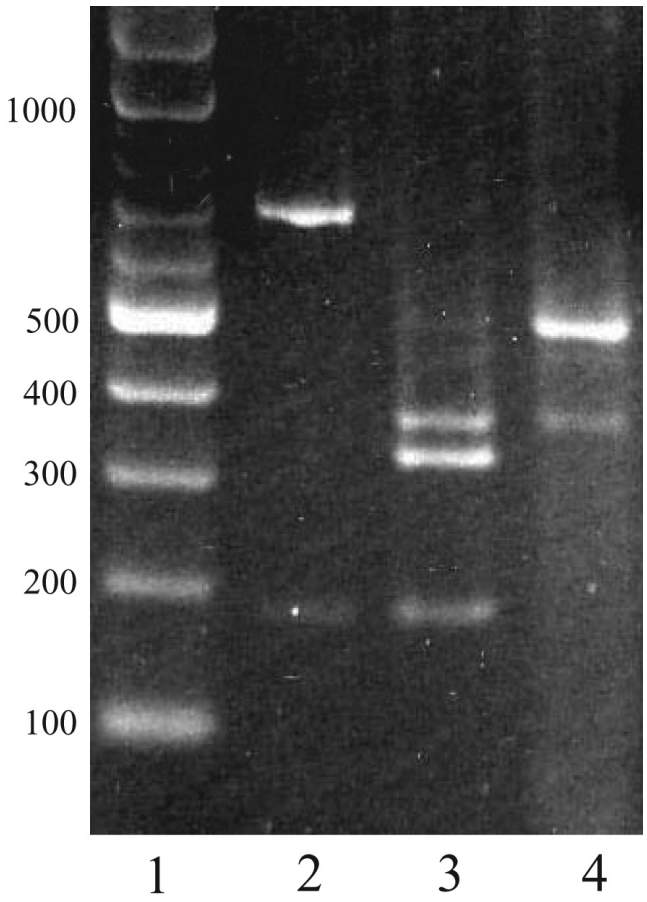

Figure 3. Restriction profiles of the COI fragments digested by *Fok* I. The first lane contains the molecular marker (100-bp DNA ladder) and the sizes are shown at the left margin. Beginning in lane 2 and moving right, species are *Moroteuthis robusta*, *M. loennbergii* and *Onykia* sp. 50-1 (= *Onykia* sp. A).

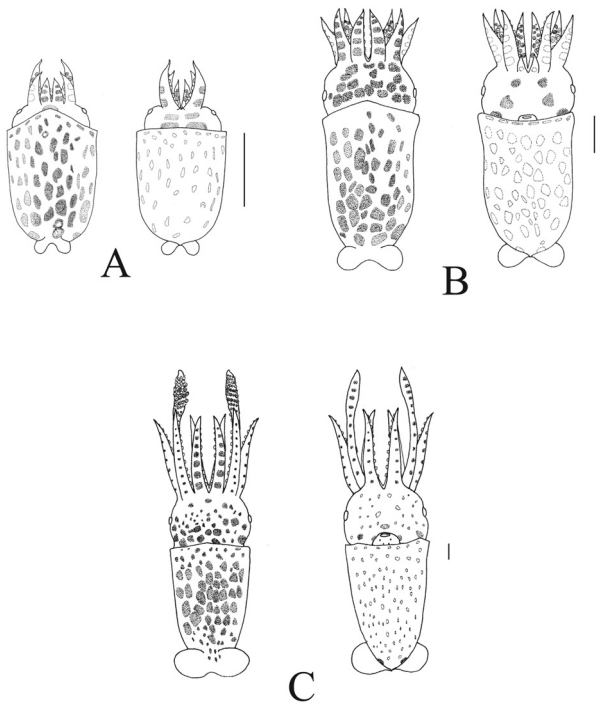

Figure 4. Dorsal and ventral view of *Onykia* (= *Moroteuthis*) *robusta*. (A) 1.8 mm DML; (B) 4.0 mm DML; (C) 7.8 mm DML. Scale bars: 1 mm.

bell-shaped, and chromatophores are distributed densely on the mantle, head, and arms and the mantle length is 1.5 times the mantle width. At 7.8 mm DML, the mantle is firm and conical and the mantle length is twice the mantle width.

The fins are attached at the posterior end of the mantle. At 1.8 mm DML, they are small, delicate, and oval and become more muscular and larger with growth. Specimens of 1.8, 4.0, and 7.8 mm DML have fin width indices of 26.8, 41.7 and 57.5%, respectively.

The head is rather cubic and sometimes half withdrawn into the mantle cavity. The head width is slightly narrower than the mantle opening at 1.8 mm DML, but is almost the same as the mantle opening in specimens >3.0 mm DML.

The funnel is relatively short and the top of the funnel reaches one-third the distance between the base of arms and the mantle margin. In specimens <4.0 mm DML, there are no chromatophores on the funnel. Two chromatophores are located near the tip of the funnel in specimens between 4.0 and 6.5 mm DML. In specimens >6.5 mm DML, 4–5 chromatophores form a transverse line on the funnel.

The arm formula is II>I>III>IV in specimens <4.5 mm DML. At 4.5–7.5 mm DML, the arm formula is II>III>I>IV, and at 8.5 mm DML, the formula is II>III>IV>I. At 1.8 mm DML, there are ten and 12 suckers on Arms I and II, respectively, and Arms III and IV are much shorter than Arms I and II. Arm II reaches about 40% of the DML. We observed 2–3 chromatophores on the aboral side of Arms I and II. At 4.0 mm DML, Arm II is about 40% of the DML. Arms I, II, and III have 4–5 chromatophores on the aboral side. A single chromatophore is present on the aboral side of Arm IV. At 7.8 mm DML, Arm II grows to 70% of the DML. Arms I, II, III, and IV have 12, 11, 10, and 10 chromatophores on the aboral side, respectively. Specimens >4.0 mm DML have chromatophores on the oral side of the arms.

At 2.0 mm DML, the tentacles are 35% of the DML and longer than the arms. Tentacle suckers are arranged in two transverse rows and six longitudinal columns, and the distal tip of the tentacles is acute. At 4.0 mm DML, the tentacle length is 40% of the DML. There are two transverse rows and six longitudinal columns of suckers in the proximal portion of the tentacles, and four transverse rows and 11 longitudinal columns in the distal portion. At 6.1 mm DML, there are ten suckers on the carpus, and four transverse rows and 14 longitudinal columns on the manus and dactylus. Marginal suckers on the manus are slightly larger than the medial ones. At 8.5 mm DML, the tentacles are of the same length as the DML, the manus suckers form four transverse rows and 23 longitudinal columns, the tentacle stalks have developed and none of the suckers have developed into hooks. Specimens of 1.8, 4.0, and 7.8 mm DML have 2, 4, and 15 chromatophores on the aboral side of the tentacles, respectively. The sucker rings of the tentacle are composed of the outer chitinous rings which possess three concentric whorls of platelets, and inner ring with the conical teeth along the distal half of the inner margin. The number of the teeth of inner rings is 3, 5 and 7 at 2.0, 6.1, 8.5 mm DML, respectively.

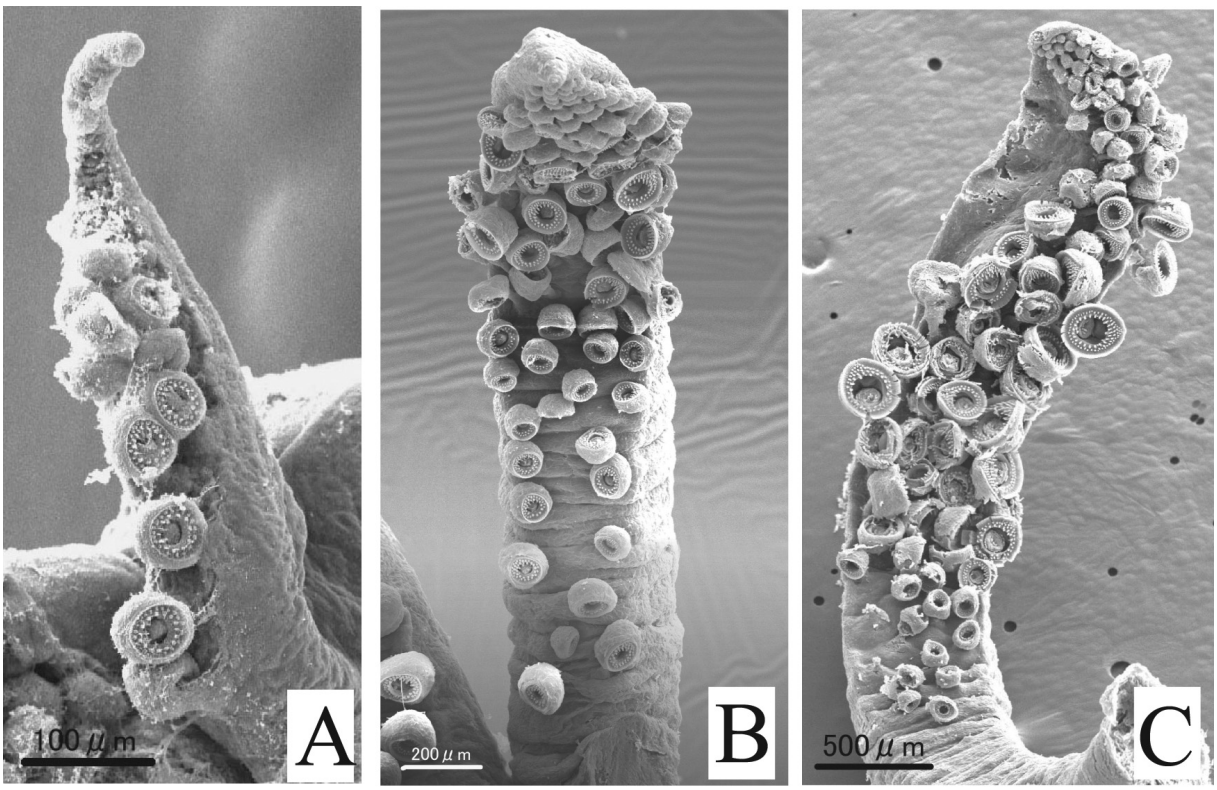

Figure 5. Scanning electron micrographs of an *Onykia robusta* tentacle. (A) 2.0 mm DML; (B) 6.1 mm DML; (C) 8.5 mm DML.

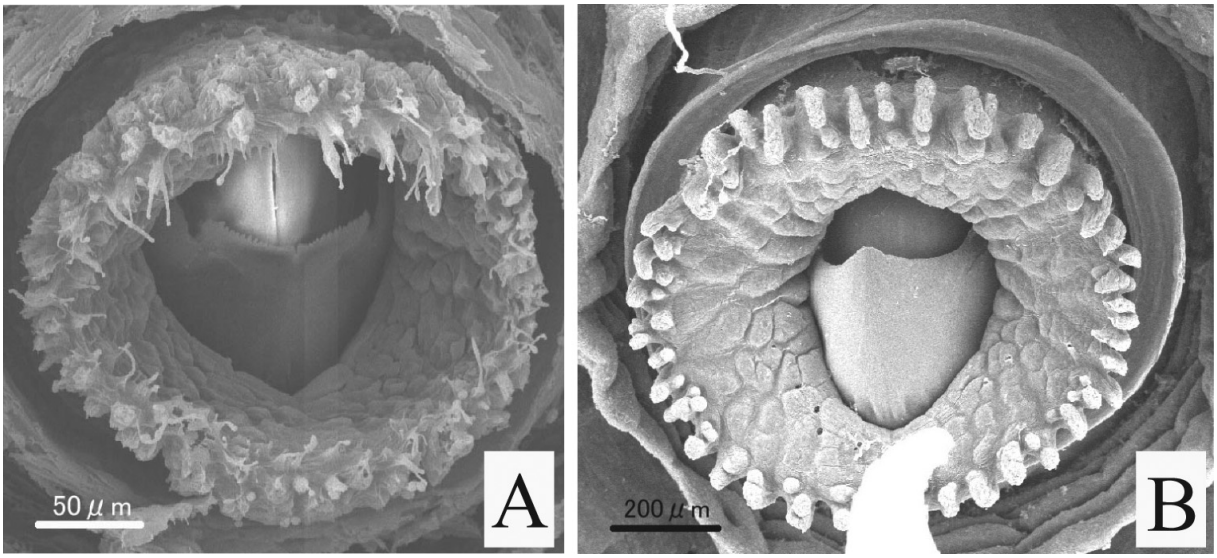

Figure 6. Scanning electron micrographs of *Onykia robusta* mouthparts. (A) 2.0 mm DML; (B) 6.1 mm DML.

The tip of the lower beak is not pointed at 2.4 mm DML. Minute dentition is present on the cutting edge of the lower beak, and lip cilia are present. At 8.5 mm DML, the rostrum of the lower beak develops. Upper beaks were not examined.

Onykia *sp. A*

The single specimen was damaged by freezing, so its arm and tentacle suckers could not be described. The morphological features are very similar to those of *O. robusta* mentioned above. The mantle is firm and conical, with the width being 70% of the DML. The funnel is relatively short, with eight chromatophores arranged in two transverse lines. There are about 250 and 150 chromatophores on the dorsal and ventral mantle, respectively. The arm formula is II>III>I>IV. The longest arm is two-thirds as long as DML, and the tentacle length is almost the same as the DML.

Distribution of paralarvae

The paralarvae of *O. robusta* were collected between 26°30' and 37°N (Figure 1) at surface temperatures between 16.7 and 24.3°C. The single paralarva of *Onykia* sp. A was collected at 28°30'N 155°W at a surface temperature of 23.3°C.

DISCUSSION

This study provides molecular evidence that most species of the genus *Onykia* described to date are juvenile stages of the genus *Moroteuthis*, as Tsuchiya & Okutani (1991) proposed. This study also provides the first description of *Onykia* (= *Moroteuthis*) *robusta* ranging in size from 1.8 to 8.5 mm DML. Our morphological investigation indicates that the paralarval stage of *O. robusta* can be divided into two distinct phases: Phase 1 (1.8–5.0 mm DML) characterized by short arms and tentacles and serrated beaks, and Phase 2 (5.1–8.5 mm DML) characterized by pointed beaks and the development of arms and tentacles. This morphological change occurring at about 5.0 mm DML may correspond to a change in feeding behaviour at this size. Similar morphological shifts associated with change in feeding behaviour have been reported in the paralarvae of many oceanic species (Wakabayashi, 2001; Wakabayashi et al., 2005).

Onykia robusta is distributed in offshore waters of the eastern and western North Pacific, from the Bering Sea and Gulf of Alaska to north-eastern Japan and southern California (Kubodera et al., 1998). Habitats of *O. robusta* may be associated with the ocean bottom, since all captures have been made by bottom trawls (Roper & Young, 1975). Mature females of *O. robusta* were collected from Santa Barbara Channel (Hochberg, 1974). Although Gilly et al. (1986) hypothesized that *Onykia* (their *Moroteuthis*) species spawn in Monterey Bay, California, based on captures of mature specimens and hatchlings in that area in September, the life cycle of *O. robusta* remains unknown. In this study, we collected paralarvae of *O. robusta* between 26°30' and 37°N in oceanic waters at surface temperatures between 16.7 and 24.3°C in November in two consecutive years. Juvenile and immature specimens of *O. robusta* have been collected between 32°30' and 35°30'N in April and May (Tsuchiya & Okutani, 1991). The spawning area may be separated from the main adult distribution area, suggesting that *O. robusta* migrates northward for feeding and returns south for spawning in autumn–winter. The very small number of mature females observed in southern waters may be due to a short spawning period or the lack of sufficient research in the central North Pacific.

Onykia sp. A could not be assigned to any *Onykia* species examined in this study. The K2P distances between *Onykia* sp. A and other *Onykia* species (0.109–0.15) were much smaller than those between *Onykia* sp. A and species of the closely related genus *Onychoteuthis* (0.204–0.215), suggesting that *Onykia* sp. A is a member of the genus *Onykia*. Although *Onykia rancureli,* distributed throughout the Indo-Pacific, may be a candidate for *Onykia* sp. A, Tsuchiya & Okutani (1991) suggested giving a new generic name to *O. rancureli* because of its distinct oval fins and gladius. Two *Onykia* species have been reported in Hawaiian waters (Bower et al., 1999). Vecchione et al. (2003) illustrated one of these species, ranging in size from 1.7 to 5.6 mm DML, which is distinct from *O. robusta*, but has a similar pattern of chromatophores on the mantle with our *Onykia* sp. A specimen.

Since *Onykia* >4.0 mm DML have numerous chromatophores on the mantle and head, this character may not be useful for species identification. Although no notable difference between *O. robusta* and *Onykia* sp. A was observed in the preliminary sorting, the results of molecular analysis allowed us to find a key character for distinguishing these species: on the funnel, *O. robusta* had 4–5 chromatophores in one transverse row, whereas *Onykia* sp. A had eight chromatophores in two rows. Since only one specimen of *Onykia* sp. A was examined, further research on more specimens is necessary to substantiate this key character.

Pfeffer (1912) and Okutani (1968) described paralarvae of *O. carriboea* <3.5 mm DML captured from the Bay of Biscay to the east coast of Patagonia in the Atlantic and off the Pacific coast of Japan, respectively, and reported these to have large, distinct, squarish chromatophores arranged in a row on the mid-dorsum of the mantle. The smallest specimen of *O. robusta* examined in the present study (1.8 mm DML) already had many chromatophores on the mantle. This difference in the chromatophore pattern suggests that *O. carriboea* (Okutani, 1968) captured from the Pacific coast of Japan is a different species from *O. robusta* examined in this study. It is difficult to decide which species of *Onykia* corresponds to the original *O. carriboea* because the type specimen of *O. carriboea* is probably not extant (Voss, 1962). Since no adult *O. carriboea* has been described in the North Pacific, the paralarva of *O. carriboea* described by Okutani (1968) may be *O. loennbergii* com. nov., considering the distribution of its adults. Paralarvae from the North and South Atlantic described as *O. carriboea* by Pfeffer (1912), might comprise more than one species. Specimens collected in the south might have been *O. robsoni* com. nov. or *O. ingens* com. nov., which are distributed in the South Atlantic. Since no adult specimens of the genus *Onykia* (*Moroteuthis*) are known from the North Atlantic, paralarvae collected in this region identified as *O. carriboea* by Pfeffer (1912) might not have been from any valid *Onykia* (*Moroteuthis*) species.

Young & Harman (1988) defined the term 'paralarva' as 'a cephalopod of the first post-hatching growth stage that is pelagic in near-surface water during the day and that has a distinctively different mode of life from that of older conspecific individuals'.The criteria that define the end of the paralarval stage in the genus *Onychoteuthis* include an abrupt increase in the number of chromatophores and development of hooks on the tentacular club (Young & Harman, 1988). Adult *Onykia* have two rows of hooks and no marginal suckers on their tentacular club. Hooks on the tentacle are well developed in specimens at 19.4 mm DML (Tsuchiya & Okutani, 1991). In the present study, no suckers had developed into hooks at 8.5 mm DML (the largest size examined), which suggests that hooks develop between 10 and 15 mm DML, when the paralarval stage ends.

Our NJ tree supported the monophyly of the genus *Onychoteuthis*. On the other hand, the monophyly of the genus *Onykia* was not supported, because '*Moroteuthis*' *knipovitchi* appears to be more closely related to the genus *Onychoteuthis* than to *Onykia* (Figure 2). Other species of *Onykia* have rugose skin, but '*M.*' *knipovitchi* is characterized by thin, smooth skin (Fillipova, 1972). Bonnaud et al. (1998) presented a NJ tree based on 16S rDNA sequence data from nine onychoteuthid species that did not support the monophyly of *Onykia* (= *Moroteuthis*), in which the relationships between '*M.*' *knipovitchi* and other congeners were similar to

our NJ tree based on COI data. Therefore, analysis of other onychoteuthid species in the remaining genera is necessary to determine the phylogenetic position of '*M.*' *knipovitchi*.

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