Molecular evidence for the existence of five cryptic species within the Japanese species of *Marphysa* (Annelida: Eunicidae) known as “Iwa-mushi”

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Abstract: Molecular phylogenetic analyses were conducted to clarify how genetically homogeneous the common Japanese species of *Marphysa* known as “Iwa-mushi” is. This is a well-known polychaete used as a fishing bait that was first described as *Marphysa iwamushi* Izuka, 1907 (type locality: Japan and Taiwan) and later synonymized to *Marphysa sanguinea* (Montagu, 1813) (type locality: England). The nucleotide sequences of a nuclear gene (18S rRNA) and two mitochondrial genes (16S rRNA and cytochrome c oxidase subunit I (COI)) were compared between specimens newly collected from 14 localities in Japan including commercially sold fishing baits and DDBJ/ENA/GenBank data for congeneric species. Our results show that the Japanese “Iwa-mushi” is not a single species but a species complex comprising five genetically well-separated clades that were tentatively designated as five undetermined species (*Marphysa* spp. A, B, C, D, and E). It is unclear whether any of these species corresponds to *M. iwamushi*. The COI nucleotide sequence of *Marphysa* sp. A was almost identical to that of *M. victori* Lavesque, Daffe, Bonifácio & Hutchings, 2017 (type locality: France) and *M. bulla* Liu, Hutchings & Kupriyanova, 2018 (type locality: China), suggesting that they are conspecific and supporting the hypothesis of Lavesque et al. (2017) that the population in France was introduced from Japan. The COI sequence of *Marphysa* sp. B corresponds to that of *M. maxidenticulata* Liu, Hutchings & Kupriyanova, 2018 (type locality: China). *Marphysa* sp. E was found only in Tokyo Bay and may be an alien species that was introduced by importing live fishing bait. Our results indicate that the European species *M. sanguinea* is not distributed in Japanese waters.

Key words: alien species, fishing bait, *Marphysa sanguinea* complex, polychaete, 16S rRNA, 18S rRNA, COI

Introduction

“Iwa-mushi” is the common Japanese name for a specific species of *Marphysa*, previously identified as *M. sanguinea* (Montagu, 1813) or *M. iwamushi* Izuka, 1907. The species commonly inhabits intertidal zones in semi-enclosed environments in and around Japan (Izuka 1912, Okuda & Yamada 1954, Imajima 1965, 2007). The Japanese name “Iwa-mushi” (Iwa:stone/rock, mushi:worm) is derived from their characteristic habit of boring into mudstones on rocky shores; however, “Iwa-mushi” is also known to inhabit burrows in the muddy and sandy sediments of tidal flats and in sediments under stones on boulder shores (Okuda & Ishikawa 1936, Ishikawa 1938, Takahashi 1960, Miura 1977, Suzuki et al. 2013).

*Marphysa iwamushi* was first described by Izuka (1907) in Japanese with the common Japanese name “Iwa-mushi.” Although Izuka (1912) described apparently the same species as *Marphysa “iwamushi”* [sic] with “n. sp.”, this name is no doubt a junior objective synonym of *M. iwamushi* Izuka, 1907. *Marphysa iwamushi* was then synonymized with
M. sanguinea by Fauvel (1933, 1936) with little explanation, but his view has been followed by most of the subsequent publications in Japan (e.g., Okuda & Ishikawa 1936, Izuka 1947, Utinomi 1956, Imajima & Hartman 1964, Imajima 1965, Miura 1977, Uchida 1992, Imajima 2007).

Marphysa sanguinea had been well-known as one of the most famous examples of a cosmopolitan polychaete species (Hutchings & Kupriyanova 2018). This is mainly because of the incomplete original description by Montagu (1813) and the subsequent synonymization of many other species with M. sanguinea without detailed explanation or revision of the type specimens (Molina-Acevedo & Carrera-Parra 2015, Zanol et al. 2016, Molina-Acevedo & Carrera-Parra 2015, Hutchings & Kupriyanova 2018). The situation has changed considerably since Hutchings and Karageorgopoulos (2003) re-described and designated a neotype for M. sanguinea based on the topotypic material and suggested that the distribution of this species is restricted to northern Europe: taxonomic revisions of species that were previously referred to as M. sanguinea have been conducted worldwide (Lewis & Karageorgopoulos 2008, Molina-Acevedo & Carrera-Parra 2015, Zanol et al. 2016, Lavesque et al. 2017, Liu et al. 2017, 2018, Elgetany et al. 2018, Wang et al. 2018).

Using RAPD-PCR, Lewis & Karageorgopoulos (2008) showed that M. sanguinea is not a cosmopolitan species but a suite of genetically differentiated species among geographically separated populations, including that in Japan. Nishi & Tanaka (2009) also suggested that “Iwa-mushi” and M. sanguinea sensu stricto are morphologically different, e.g., in chaetal morphology, and the two are possibly different species. In response to these studies, the name M. iwamushi (or M. cf. iwamushi) has been used again as the specific name for “Iwa-mushi” in Japan (e.g., Nishi et al. 2009, Suzuki et al. 2013, Saito et al. 2014, Masumoto et al. 2017).

However, using M. iwamushi for “Iwa-mushi” is still problematic because (1) the identity of M. iwamushi remains unknown owing to the incomplete original descriptions of Izuka (1907, 1912) with regard to important morphological characteristics in the current taxonomy of Marphysa (Glasby & Hutchings 2010, Molina-Acevedo & Carrera-Parra 2015), and (2) there is a possibility that the present Japanese “Iwa-mushi” population may comprise several distinct species.

Another problem pointed out by Suzuki et al. (2013) is the possible presence of established populations of non-indigenous cryptic species of Marphysa in Japan brought by the import of live fishing baits. In Japan, at least two species of Marphysa were heavily exploited by local fishermen as fish bait (Izuka 1912, Ishikawa 1938, Takahashi 1960, Imajima 1971); however, the exploitation has steadily decreased and cultivation of the worms has been limited owing to several technical difficulties (Kitamori 1972, Imai 1982, Maeda & Tominaga 2008). Import of live Marphysa from Korea to Japan as fishing baits started in 1969; since the 1990s, Japan has shifted import of these worms from China (Weekly Sunday Fishing 1997, Hayashi 2001, Saito et al. 2011, Saito 2016). Japanese native “Iwa-mushi” and Marphysa imported from Korea and China have been heretofore considered as the same species under the names of M. sanguinea or M. cf. iwamushi (Nishi & Kato 2004, Nishi 2005, Saito et al. 2011, 2014, Saito 2016).

Recently, Liu et al. (2017, 2018) and Wang et al. (2018) described five and one new species of Marphysa, respectively, from China. The following eight species of Marphysa are currently recognized from intertidal zones in China: M. bulla Liu, Hutchings & Kupriyanova, 2018, M. digibranchia Hoagland, 1920, M. hongkongensa Wang, Zhang & Qiu, 2018, M. maxidenticulata Liu, Hutchings & Kupriyanova, 2018, M. multipectinata Liu, Hutchings & Sun, 2017, M. orientalis Treadwell, 1936, M. tribranchiata Liu, Hutchings & Sun, 2017, and M. tripectinata Liu, Hutchings & Sun, 2017. Marphysa sinensis Monro, 1934 was transferred to genus Paucibranchia by Molina-Acevedo (2018). Although M. bulla, M. maxidenticulata, and M. tripectinata were reported to be exploited as fishing baits in China (Liu et al. 2017, 2018), the number of species imported by Japan remains unknown. The intentional import of live fishing baits and their increasing use in recreational fishing contributes to introduction of non-indigenous species (Nishi & Kato 2004, Cole et al. 2018, Pombo et al. 2018) that may be difficult to detect because of the morphological similarity between the native and non-indigenous species (e.g. Nishizawa et al. 2014).

In this study, we conducted molecular analyses on “Iwa-mushi” worms, which were newly collected from various regions or purchased at fishing stores in Japan, to reveal whether this species of Marphysa is a complex consisting of several cryptic species.

Materials and Methods

Specimens of Marphysa referable to “Iwa-mushi” were collected from intertidal to subtidal zones with rocky, boulder, sandy, or muddy substrata in 14 Japanese localities (Table 1, Fig. 1). A part of specimens was obtained by purchasing the fishing baits that were collected by local fishermen and regionally sold under the local market names: “Maeba” in Sendai and “Benten-jamushi” in Hama-matsu (Table 1). All specimens were fixed and preserved in 99% ethanol, and stored at 4°C. Preliminary morphological observations of the preserved specimens were conducted under a stereomicroscope (Olympus SZX 9).

Genomic DNA was extracted from a small piece of tissue (branchiae, pygidial cirri, or body wall) from each specimen by grinding and heating at 95°C for 20 min in 50 μL TE buffer (pH 8.0) with 10% Chelex 100 (Bio-Rad Laboratories, Hercules, CA; Richlen & Barber 2005). Depending on the DNA concentration, undiluted or 10-fold diluted DNA extract was used as a template for polymerase chain reaction (PCR). Partial sequences of nuclear 18S rRNA (18S) and mitochondrial 16S rRNA
(16S) and cytochrome c oxidase subunit I (COI) genes were amplified by PCR using the following primer pairs: 18S-1F1/18S-1R632, 18S-2F576/18S-2R1209, and 18S-3F1129/18S-R1772 for 18S, Mar16S-F/Mar16S-R for 16S, and MarCOI-F/polyHCO for COI (Table 2). Mar16S-F/Mar16S-R and MarCOI-F were designed for Marphysa in this study and are modifications of the 16Sar/16Sbr (Palumbi et al. 1991) and ACOIAF primers (Colgan et al. 2001), respectively. PCR was performed in a 15 µL reaction mixture containing 1.0–2.0 µL of template DNA, 1 µL of each primer pair. The PCR cycling conditions were 28–36 cycles of 98°C for 10 s, 60°C for 5 s, and 68°C for 1 s for 18S; 32–36 cycles of 98°C for 10 s, 52–57°C for 5 s, and 68°C for 1 s for 16S; and 32–38 cycles of 98°C for 10 s, 40–43°C for 5 s, and 68°C for 1 s for COI. The PCR products were purified using ExoSAP-IT (Affymetrix, Cleveland, OH) and sequenced by Eurofins Genomics (Tokyo, Japan). The forward and reverse complementary sequences and contigs were assembled using GeneStudio ver. 2.2.0.0 (GeneStudio, Inc. Suwanee, GA). The gene sequence data obtained in this study have been deposited in the DDBJ/ENA/GenBank databases with accession numbers LC467718–LC467789 (Table 3).

To reconstruct the molecular phylogeny, sequences of the 18S, 16S, and COI genes were aligned with the sequences of other species of Marphysa, Paucibranchia, and outgroup taxa available in the DDBJ/ENA/GenBank databases (Table 3) using the MAFFT online service ver. 7 with the L-INS-i algorithm (Katoh et al. 2019). Ambiguously aligned regions were eliminated using the Gblocks server ver. 0.91b (Castresana 2000). The final lengths of the aligned sequences were 1762 bp for 18S, 519 bp for 16S, and 663 bp for COI. Four phylogenetic trees were constructed based on the concatenated sequences of 18S, 16S, and COI, and the sequences of each gene region separately using maximum likelihood (ML) methods. ML analyses were performed using RAxML ver. 8.0.26 (Stamatakis 2014) as implemented in raxmlGUI ver. 1.5 (Silvestro & Michalak 2012) under the GTR+GAMMA+I model. The robustness of the ML trees was evaluated by rapid bootstrap analysis with 1,000 replicates.

The COI sequences labeled as Marphysa sp. 1, 2, and 3 (DDBJ/ENA/GenBank accession numbers KY323709, KY328276, and KY328279) were referred to as M. tripectinata, M. bulla, and M. maxidenticulata, respectively, in

| Table 1. List of specimens collected including the sampling localities, environment, habitat of the specimens, date of collection or purchase, and number (n) of specimens used for molecular analyses. |
|-----------------|-----------------|-----------------|-----------------|
| Species         | Market name     | Locality        | Geolocation     |
| Marphysa sp. A  | Moune Bay       | 38°53′42″N,     |
|                 |                 | 141°37′34″E     |
|                 | Mangoku-ura Inlet| 38°25′06″N,     |
|                 |                 | 141°22′52″E     |
|                 | Soukanzan, Matsushima Bay | 38°21′08″N,     |
|                 |                 | 141°3′32″E      |
| Maeba           | Shichigahama, Sendai Bay | -              |
|                 |                 |                  |
|                 | Ninzaki, Nanao Bay | 37°12′15″N,     |
|                 |                 | 136°55′07″E     |
|                 | Moroiso Bay     | 35°09′29″N,     |
|                 |                 | 139°36′44″E     |
|                 | Ena Bay         | 35°08′40″N,     |
|                 |                 | 139°39′50″E     |
| Benten-         | Lake Hamana     | -               |
| jamushi         |                 |                  |
| Marphysa sp. B  | Arao tidal flat, Ariake Sea | 32°57′32″N,     |
|                 |                 | 130°25′41″E     |
| Marphysa sp. C  | Samegawa River tidal flat | 36°54′34″N,     |
|                 |                 | 140°49′00″E     |
|                 | Tsurugizaki, Sagami Bay | 35°08′26″N,     |
|                 |                 | 139°40′35″E     |
| Marphysa sp. D  | Katsuozaki, Toyama Bay | 37°08′56″N,     |
|                 |                 | 137°03′10″E     |
| Marphysa sp. E  | Sanbanze Ocean Park, Tokyo Bay | 35°40′02″N,     |
|                 |                 | 139°58′14″E     |
|                 | Yoro River tidal flat, Tokyo Bay | 35°32′37″N,     |
|                 |                 | 140°03′56″E     |
|                 |                 |                  |
| a: mesh bags for spat collection of Manila clam Ruditapes philippinarum (see Toba et al. 2017) |
accordance with Wang et al. (2018). Although the deposited sequences were not included in the original description by Liu et al. (2017, 2018), correspondence between the sequences and species names were confirmed by the first author (Zhi Wang, personal communication). The DDBJ/ENA/GenBank sequence labeled as *M. sanguinea* (KF733802) was referred to as *Marphysa* sp. since the sequence is divergent from the authoritative sequences of *M. sanguinea* (GQ478157 and GQ497547; Zanol et al. 2010); this species could have been misidentified (Lavesque et al. 2019). Some DDBJ/ENA/GenBank sequences labeled as *Marphysa* species were referred to as *Nicidion* or *Pauci-

Table 2. List of primers used for PCR amplification in this study.

| Gene    | Primer        | Direction | Sequence (5′–3′)                  | Reference                  |
|---------|---------------|-----------|-----------------------------------|----------------------------|
| 18S rRNA| 18S-1F1       | Forward   | AACCTGGTTGATYCTGCCAG              | Nishitani et al. (2012)    |
|         | 18S-1R632     | Reverse   | ACTACGAGCTTTTTAACYGCARC           | Nishitani et al. (2012)    |
|         | 18S-2F576     | Forward   | GGTAATCCAGCTCYAATRG               | Nishitani et al. (2012)    |
|         | 18S-2R1209    | Reverse   | AAGTTTYCCCGTGTTGARTC              | Nishitani et al. (2012)    |
|         | 18S-3F1129    | Forward   | GCTGAAACTTAAAGRAATTGACGG          | Nishitani et al. (2012)    |
|         | 18S-R1772     | Reverse   | TCACCTACGGAAAACCTTTGTTACG         | Nishitani et al. (2012)    |
| 16S rRNA| Mar16S-F      | Forward   | CTGTITTWCAAAAAACATYGCC            | This study                 |
|         | Mar16S-R      | Reverse   | CGGTCTGAACTCAGKTCA                | This study                 |
| COI     | MarCOI-F      | Forward   | TYWACAAAYCAYAAAGAYATTGG           | This study                 |
|         | polyHCO       | Reverse   | TAMACTTCWGGGTGACAAAATCA           | Carr et al. (2011)         |

Fig. 1. Map showing sampling localities of *Marphysa* in Japan (A) and detailed maps for northeastern Japan (B), the Noto Peninsula (C), and the vicinity of Tokyo Bay (D). Symbols indicate the sampling localities of each species of *Marphysa* analyzed in this study.
Results

Molecular phylogenetic analysis of the concatenated sequences revealed that the "Iwa-mushi" samples were divided into five genetically distinct clades (CL1–CL5, Fig. 2A) corresponding to five species namely Marphysa spp. A–E as described below. Interspecific phylogenetic relationships were ambiguous due to low bootstrap values (below 50%) at most of the higher internal nodes (Fig. 2A–D).

Table 3. List of terminal taxa used in the phylogenetic analyses with their sampling country, DDBJ/ENA/GenBank accession numbers, and references. The gene sequences obtained from this study are highlighted in boldface type.

| Classification | Species | Country          | DDBJ/ENA/GenBank accession number | Reference |
|----------------|---------|------------------|-----------------------------------|-----------|
|                | Marphysa |                 |                                   |           |
|                | sp. A    | Japan            | LC467718–LC467725                 | This study |
|                |          |                  | LC467742–LC467749                 |           |
|                |          |                  | LC467766–LC467773                 |           |
|                | sp. B    | Japan            | LC467726–LC467730                 | This study |
|                |          |                  | LC467750–LC467754                 |           |
|                | sp. C    | Japan            | LC467731–LC467734                 | This study |
|                |          |                  | LC467755–LC467758                 |           |
|                | sp. D    | Japan            | LC467735                          | This study |
|                |          |                  | LC467759                          |           |
|                | sp. E    | Japan            | LC467736–LC467741                 | This study |
|                |          |                  | LC467760–LC467765                 |           |
|                |          |                  | LC467784–LC467789                 |           |
|                |          |                  |                                   |           |
|                | Marphysa  |                 |                                   |           |
|                | aegypti  | Egypt            |                                   |           |
|                | bifurcata| Australia         | -                                 |           |
|                | brevetentaculata | Mexico | GQ497503–GQ478158 |           |
|                | bulla    | (as Marphysa sp. 2) | China - GQ478162                 |           |
|                | californica | USA           | GQ497507–GQ478162                |           |
|                | corallina | South Africa     | -                                 |           |
|                | fauchaldi| Australia         | -                                 |           |
|                | gravelyi | India            | -                                 |           |
|                | hongkongensa | Hong Kong  | -                                 |           |
|                | kristiani| Australia         | -                                 |           |
|                | maxidenticulata | (as Marphysa sp. 3) | China - GY328279                 |           |
|                | mullawa  | Australia         | -                                 |           |
|                | pseudossilosa | Australia | -                                 |           |
|                | regalis  | Brazil            | GQ497510–GQ478165                |           |
|                | sanguinea| France            | GQ497502–GQ478157                |           |
|                | tamurai  | -                 | GQ497504–GQ478159                |           |
|                | tripectinata | (as Marphysa sp. 1) | China - GY323709                 |           |
|                | victori  | France            | -                                 |           |
|                | viridis  | Brazil            | GQ497508–GQ478163                |           |
|                | sp. (as M. sanguinea) | China | -                                 |           |
|                |         |                  |                                   |           |
|                | Paucibranchia |                 |                                   |           |
|                | bellii (as M. bellii) | France, Spain | AF412789–AY838835                |           |
|                |         |                  |                                   |           |
|                | Paucibranchia |                 |                                   |           |
|                | cf. bellii (as M. cf. bellii) | Greece | GQ497511–GQ478157                |           |
|                | disjuncta (as M. disjuncta) | USA | GQ497504–GQ478159                |           |
|                | fallax (as M. fallax) | Italy | GQ497505–GQ478160                |           |
|                |         |                  |                                   |           |
|                | Nicidion |                  |                                   |           |
|                | angeli (as M. angeli) | Brazil | GQ497506–GQ478164                |           |
|                |         |                  |                                   |           |

branchia species according to Molina-Acevedo & Carrera-Parra (2017) and Molina-Acevedo (2018).

(I) Marphysa sp. A (Fig. 3A, B)

Eight specimens of Marphysa sp. A were collected from various localities along the coasts of the Pacific Ocean and Sea of Japan (Fig. 1). These specimens can be found in muddy and sandy sediments under stones on tidal flats and boulder shores, inside mudstones, and occasionally from burrows in the muddy sediments in eelgrass beds of Zostera marina Linnaeus, 1753 (Table 1).

Body lengths of the preserved specimens ranged between 5.5–11 cm with 178–307 chaetigers in complete specimens and 3–8 cm with 106–211 chaetigers in incomplete anterior or posterior fragments. Bodies of the worms were beige to reddish brown with dark brown mottled pig-
mentation and increasing iridescence towards the anterior in both live and preserved specimens (Fig. 3A, B). The specimens had limbate capillaries and compound spinigers in neurochaetae without compound falcigers.

Marphysa sp. A was categorized in CL1 together with M. bulla and M. victori Lavesque, Daffe, Bonifácio & Hutchings, 2017 (Fig. 2A). The COI sequences of Marphysa sp. A shared 99.5–100% identity with those of M. bulla from China and M. victori from France (Table 3, Fig. 2D). Furthermore, the 16S sequences of Marphysa sp. A and those of the paratypes of M. victori (DDBJ/ENA/GenBank accession numbers MG385000 and MG385001) shared 100% identity (Fig. 2C).

(2) Marphysa sp. B (Fig. 3C, D)

The five specimens of Marphysa sp. B were collected...
Cryptic species of *Marphysa* from burrows in the muddy sand sediment in tidal flats off Arao in the Ariake Sea, Kyushu, southern Japan (Table 1, Fig. 1).

Body lengths of the preserved specimens were 11–12 cm with 265–316 chaetigers in complete specimens and 7–10 cm with 69–113 chaetigers in incomplete anterior fragments. The worms looked beige to reddish pink with increasing iridescence towards the anterior in both live and preserved specimens (Fig. 3C, D). The specimens had limbate capillaries and compound spinigers in neurochaetae without compound falcigers.

CL2 comprised *Marphysa* sp. B, *M. maxidenticulata*, and *Marphysa* sp. (Fig. 2A). The COI sequences of *Marphysa* sp. B shared 99.8–100% identity with those of *M. maxidenticulata* and *Marphysa* sp. (KF733802) from China (Table 3, Fig. 2D).

(3) *Marphysa* sp. C (Fig. 3E, F) CL3 consisted of four samples of *Marphysa* sp. C found under stones in the sandy tidal flats in the estuarine region of the Samegawa River, southern Tohoku District and inside mudstones on the rocky shore of Tsurugizaki, Miura Peninsula, Sagami Bay (Table 1, Fig. 1).

Body lengths of the preserved specimens ranged between 5–16 cm with 221–422 chaetigers in complete specimens and 3–4 cm with 115–124 chaetigers in incomplete anterior fragments. Body color of the worms was pinkish orange to beige with dark brown mottled pigmentation and increasing iridescence towards the anterior in both live and preserved specimens. Anterior mottled pigmentation was faint in a few small individuals (Fig. 3E, F). The specimens had limbate capillaries and compound spinigers in neurochaetae without compound falcigers.

CL3 consisted exclusively of *Marphysa* sp. C and formed a sister group with *M. viridis* Treadwell, 1917 (Fig. 2A). There were no 16S and COI sequences available in DDBJ/ENA/GenBank that were identical to those of the samples (Fig. 2C, D). Although their 18S sequences were completely identical (Fig. 2B), *Marphysa* sp. B, *Marphysa* sp. C, and *M. viridis* from Brazil (Table 3) were clearly distinguishable by their 16S and COI sequence analyses (Fig. 2C, D).

(4) *Marphysa* sp. D (Fig. 3G) A single incomplete posterior fragment of *Marphysa* sp. D was collected from the sandy subtidal zone in Katsuwazaki, Toyama Bay, Sea of Japan (Table 1, Fig. 1).

The length and width of the preserved posterior fragment was ca. 2 cm and 4 mm, respectively, with 71 chaetigers and the color was yellowish white (Fig. 3G). The specimen had limbate capillaries and compound spinigers in neurochaetae without compound falcigers.

CL4 was only represented by *Marphysa* sp. D and there were no sequences available in DDBJ/ENA/GenBank data.
bases that matched this species (Fig. 2).

(5) *Marphysa* sp. E (Fig. 3H)

Six specimens, all incomplete posterior fragments of *Marphysa* sp. E, were collected from burrows in the muddy sand tidal flat in Tokyo Bay (Sanbanze Ocean Park and mouth of the Yoro River) (Table 1, Fig. 1).

The anterior-most part of this worm could not be collected owing to its long body (>30 cm) and deep burrowing habit. The length of the posterior fragments analyzed were between 3–8 cm with 72–230 chaetigers. The live and preserved worms were reddish pink and yellowish white or beige, respectively (Fig. 3H). The specimens have limbate capillaries in neurochaetae without compound chaetae. Conical mounds of fecal pellets excreted by this worm were frequently observed at the collection sites in Tokyo Bay (Fig. 3I).

*Marphysa* sp. E was equivalent to CL5 that was the sister group of *M. mossambica* (Peters, 1854) (Fig. 2A). There were not any sequences that were identical to those of CL5 in the DDBJ/ENA/GenBank databases (Fig. 2).

**Discussion**

Molecular analyses in this study clearly distinguished five clades within the Japanese species of *Marphysa* known as "Iwa-mushi." These five clades were regarded as different species since the genetic differentiation among the clades were comparable to that among several nominal species previously known in *Marphysa* (Fig. 2). Although the COI sequences of CL1 and CL2 shared similarity with identified sequences from DDBJ/ENA/GenBank, we refer to the five clades as undetermined *Marphysa* spp. A–E. To accurately identify these species, detailed morphological analyses and comparisons of these five species are required along with establishing the identity of *M. iwamushi* based on the re-description of its name-bearing types.

According to Glasby & Hutchings (2010), the species belonging to *Marphysa* are classified into five informal groups based on the following type of compound chaetae present in the subacicular neurochaetae, i.e. the Mossambica-group (=Group A sensu Fauchald 1970) includes species lacking any compound chaetae; the Sanguinea-group (=Group B) includes species with compound spinigers only; the Aeana-group (=Group C) includes species with compound falcigers only; the Belli-group (=Group D) includes species with both compound falcigers and spinigers; and the Teretiuscula-group includes species with compound spinigers restricted to the anterior chaetigers only. Based on this categorization, *Marphysa* spp. A–D and *Marphysa* sp. E were classified into the Sanguinea-group and the Mossambica-group, respectively, even though the *Marphysa* sp. D–E samples were incomplete posterior fragments. Besides *M. sanguinea* and *M. iwamushi*, the following seven species of *Marphysa* are currently recognized in Japan: *M. bifurcata* Kott, 1951, *M. depressa* (Schmarda, 1861), *M. macintoshi* Crossland, 1903, *M. mortenseni* Monro, 1928, *M. tamurai* Okuda, 1934, and *Marphysa* sp. B and C sensu Imajima, 2007 (Okuda 1934, Iwase et al. 1990, Uchida 1992, Imajima 2006, 2007). *Marphysa bellii* (Audouin & Milne Edwards, 1833), *M. confer-ta* Moore, 1911, *M. disjuncta* Hartman, 1961, *M. kinbergii* McIntosh, 1910, *M. stragulum* (Grube, 1878), and *Marphysa* sp. A sensu Imajima, 2007 previously recorded from Japan (Imajima 1970, 2006, 2007, 2011, Miura 1977, 1979) were recently transferred to genus *Paucibranchia* (Molina-Acevedo 2018). Among above seven *Marphysa* species in Japan, *M. macintoshi* and *M. tamurai* belong to the Sanguinea-group. Since they possess a prostomium with a very faint middle notch (Okuda 1934, Iwase et al. 1990), they are at least different from *Marphysa* spp. A–C with the distinct middle notch-containing prostomium (Fig. 3A–F). Other Japanese species *M. bifurcata* and *M. mortenseni* belong to the Aeana-group; *M. depressa* and *Marphysa* sp. C sensu Imajima, 2007 to the Belli-group; and *Marphysa* sp. B sensu Imajima, 2007 to the Mossambica-group. Although they both belong to the Mossambica-group, it is unclear whether *Marphysa* sp. E (from our study) and *Marphysa* sp. B sensu Imajima, 2007 are the same species.

The COI sequences of *Marphysa* sp. A were almost identical to those of *M. bulla* and *M. victori* obtained from the DDBJ/ENA/GenBank databases (Fig. 2), suggesting that they may be the same species. This presumption was also supported by our 16S sequence data that showed complete identity between *Marphysa* sp. A and *M. victori*. *Marphysa bulla* and *M. victori* were described based on specimens collected from the Yellow Sea, China by Liu et al. (2018) and Arcachon Bay, France by Lavesque et al. (2017), respectively. These species have only been found in their respective type localities inhabiting rock crevices (*M. bulla*) and around oyster reefs, abandoned oyster farms, or galleries in old pieces of driftwood (*M. victori*). Both species were morphologically recognized to belong to the Sanguinea-group similar to *Marphysa* sp. A; however, in contrast to the molecular analyses, the descriptions of *M. bulla* and *M. victori* indicate some morphological differences between these two species, e.g., in maxillary formula, presence of subacicular hooks, and the number of types of pectinate chaetae (Lavesque et al. 2017, Liu et al. 2018).

In the original description of *M. victori*, it was hypothesized that this species may not be indigenous to France and could have been transferred by "hitchhiking" on exotic Pacific oyster *Crassostrea gigas* (Thunberg, 1793) that were transported to Arcachon Bay (type locality of *M. victori*) from Japan or Canada (Lavesque et al. 2017) as is the case with several non-indigenous species (Gouillieux et al. 2016). After the mass mortality of the Portuguese oyster *Crassostrea angulata* ( Lamarck, 1819) in France, restoring oyster culturing involved the mass export of oyster spat (*C. gigas*) from Japan, especially from Sendai Bay, to France between 1969 and 1979 (Koganezawa 1984, Koike 2015). This oyster spat was also introduced into Arcachon Bay.
between 1971 and 1975 (Grizel & Héral 1991). Interestingly, Marphysa sp. A was collected from Sendai Bay and its branches, i.e. Mangoku-ura Inlet and Matsushima Bay (Table 1). Our results support the hypothesis by Lavesque et al. (2017) that the M. victori population in France was introduced from Japan.

Marphysa sp. E excretes fecal pellets on the surface of tidal flats and forms distinctive fecal mounds (Fig. 3I). These fecal mounds are frequently found in the Sanbanze Ocean Park and mouth of the Yoro River in Tokyo Bay. The fecal pellets have been used to study the chemistry of organic environmental toxicants (identified as M. sanguinea; Onozato et al. 2010, 2012, Nishigaki et al. 2013). Owing to their unusual distribution, we cannot eliminate the possibility that Marphysa sp. E is a non-indigenous alien species introduced via imported live bait: this species was only found in Tokyo Bay (Table 1) that is one of the biggest “melting pots” of alien species in Asia (Asakura 1992, Iwasaki 2006, Abe et al. 2019). To ascertain the indigeneity of this species, it is important to compare its morphology and gene sequences with those of the species of Marphysa imported from China as live fishing bait. The results of this study indicate that the confusion in taxonomy of Marphysa probably arises from the intentional and unintentional introduction of exotic species by transporting oyster and import of live fishing bait.

Marphysa sp. B and M. maxidenticulata are likely to be the same species since their COI sequences were almost identical. Marphysa maxidenticulata was originally categorized in the Belli-group (Liu et al. 2018). However, Wang et al. (2018) re-categorized this species to the Sanguinea-group because the short-bladed compound falcigers (sensu Liu et al. 2018) in M. maxidenticulata (as well as in M. orientalis shown by Liu et al. 2018) were re-interpreted as compound spinigers: spinigers and falcigers in Marphysa are recognized by unidentate sharp tips and bidentate hooded tips, respectively (Zhi Wang, personal communication). Their interpretation is consistent with our results; Marphysa sp. B is a part of the Sanguinea-group. The current distribution of M. maxidenticulata is restricted to Caofoedian (Bohai Sea) and the Qingdao coast (Yellow Sea), north China (Liu et al. 2018). Our study may expand the known distribution range of M. maxidenticulata to include the Ariake Sea, south Japan. The distribution of Marphysa sp. B suggests that it may be a potentially relict species that was isolated from the original Asian continental coast population by geohistorical events and has existed only in the Ariake Sea, a semi-closed shallow sea with a large tidal flat and macro-tidal environment similar to the Bohai and Yellow Sea (Sato & Takita 2000, Sato 2010, 2017).

Although we do not know whether any of the five undetermined species correspond to M. iwamushi, we can infer from their habitats and sampling localities that Marphysa sp. A is most likely to be M. iwamushi. In the original description, Izuka (1907) mentioned that M. iwamushi is found in various regions of Japan and the Pescadores (=Penghu) Islands, Taiwan and lives inside intertidal muddy sand and soft rocks. Izuka (1912) also stated that M. iwamushi is “one of the most common littoral annelids in Japan” (p. 131) and “found usually burrowing in tertiary rocks, but sometimes in sandy flats” (p. 133). In our study, we found Marphysa sp. A often burrowed into mudstones as well as muddy or muddy sand sediment especially under stones half-buried in tidal flats and inhabited various regions in Japan. Although Marphysa sp. C also burrows into mudstones, as compared to Marphysa sp. A this species was less frequently collected in our study (Table 1) and seemed to prefer habitats facing the open ocean: most of the benthic species living around the collection site of Marphysa sp. C in tidal flats in the Samegawa River estuary were those typically found in rocky shores in outer bay environments (Kanaya et al. 2019), and Tsurugizaki at the southeastern end of the Miura Peninsula is also a rocky shore environment facing the open ocean (Fig. 1). To determine whether Marphysa sp. A and M. iwamushi are the same species, it is imperative to re-describe the type specimens of M. iwamushi deposited in the University Museum, the University of Tokyo (Nishi & Tanaka 2011) and compare their morphologies. However, this is beyond the scope of this study and will be addressed in the future.

Note that molecular analyses showed that none of the sequences of Marphysa obtained from our study matched with the authoritative sequences of M. sanguinea from France (Zanol et al. 2010). Thus, there is currently no evidence to support the presence of M. sanguinea in Japan, further strengthening the validity of M. iwamushi as a different species.

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