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# Molecular identification and larval morphology of spionid polychaetes (Annelida, Spionidae) from northeastern Japan

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## Abstract

Planktonic larvae of spionid polychaetes are among the most common and abundant group in coastal meroplankton worldwide. The present study reports the morphology of spionid larvae collected mainly from coastal waters of northeastern Japan that were identified by the comparison of adult and larval 18S and 16S rRNA gene sequences. The molecular analysis effectively discriminated the species. Adult sequences of 48 species from 14 genera (*Aonides* Claparède, 1864; *Boccardia* Carazzi, 1893; *Boccardiella* Blake & Kudenov, 1978; *Dipolydora* Verrill, 1881; *Laonice* Malmgren, 1867; *Malacoceros* Quatrefages, 1843; *Paraprionospio* Caullery, 1914; *Polydora* Bosc, 1802; *Prionospio* Malmgren, 1867; *Pseudopolydora* Czerniavsky, 1881; *Rhynchospio* Hartman, 1936; *Scolelepis* Blainville, 1828; *Spio* Fabricius, 1785; *Spiophanes* Grube, 1860) and larval sequences of 41 species from 14 genera (*Aonides*; *Boccardia*; *Boccardiella*; *Dipolydora*; *Laonice*; *Paraprionospio*; *Poecilochaetus* Claparède in Ehlers, 1875; *Polydora*; *Prionospio*; *Pseudopolydora*; *Rhynchospio*; *Scolelepis*; *Spio*; *Spiophanes*) of spionid polychaetes were obtained; sequences of 27 of these species matched between adults and larvae. Morphology of the larvae was generally species-specific, and larvae from the same genus mostly shared morphological features, with some exceptions. Color and number of eyes, overall body shape, and type and arrangement of pigmentation are the most obvious differences between genera or species. The morphological information on spionid larvae provided in this study contributes to species or genus level larval identification of this taxon in the studied area. Identification keys to genera and species of planktonic spionid larvae in northeastern Japan are provided. The preliminary results of the molecular phylogeny of the family Spionidae using 18S and 16S rRNA gene regions are also provided.

**Keywords**
Larval identification, meroplankton, molecular identification, phylogeny, planktonic larvae, 16S rRNA, 18S rRNA

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## Introduction

Many marine invertebrates including polychaetes pass through a planktonic larval phase during their early life history. As such, planktonic larvae derived from the benthic polychaetes are one of the most numerous and diverse groups of coastal zooplankton (Thorson 1946; Hansen 1999; Omel'yanenko and Kulikova 2002; Blake 2017). Polychaetes are often represented in coastal benthic fauna with high species richness all over the world, and members of this group play a large role in the functioning and food webs of marine ecosystems (Aller 1982; Laffaille et al. 2005; Tomiyama et al. 2005, 2007). However, the field study of larval ecology has been restricted because of difficulties in larval identification, which is largely caused by the radical morphological differences between larval and adult stages, lack of diagnostic key characters of larvae, and lack of information on larval forms of many species as is also the case for other marine invertebrates (Branscomb and Vedder 1982; Shanks 1986; Levin 1990). Extensive efforts to describe planktonic polychaete larvae from coastal waters have been performed for species from European and American waters (Blake 2017, and the references cited therein). However, most of these studies are very limited regarding other areas, although Carrasco (1976) and Wu et al. (1978) described polychaete larvae of many species from Chilean and Chinese waters, respectively. In Japan, the larval development and morphology of some polychaete species have been studied (e.g., Izuka 1912: Nereididae Blainville, 1818; Okada 1930: Syllidae Grube, 1850; Okuda 1946: 9 families; Imajima 1959: Spionidae Grube, 1850; Choe 1960: Eunicidae Berthold, 1827; Yamaji 1966: 16 families; Tokioka 1970: Amphinomidae Lamarck, 1818; Imai 1975, 1982: Eunicidae; Miura and Kajihara 1981: Serpulidae Rafinesque, 1815; Yokoyama 1981, 1996: Spionidae; Sasaki and Brown 1983: Saccocirridae Bobretzky, 1872; Yokouchi 1985, 1988: 27 families; Sato and Tsuchiya 1991: Nereididae; Nishi and Yamasu 1992a, 1992b, 1992c, 1992d: Serpulidae; Yokouchi and Yokouchi 1997: 23 families; Koya et al. 2003: Nereididae; Tosuji and Sato 2006: Nereididae; Kondoh et al. 2017: Spionidae; Kan et al. 2020: Nereididae), and some field ecological investigations of polychaete larvae have been conducted (Yokouchi 1984, 1991; Yokoyama 1990, 1995; Abe et al. 2011, 2014; Kan et al. 2020).

Spionidae is one of the largest taxa of polychaete annelids and currently comprises more than 500 nominal species belonging to approximately 38 genera (Radashevsky 2012; Read and Fauchald 2020, excluding *Poecilochaetus* Claparède in Ehlers 1875 and *Trochochaeta* Levinsen, 1883). Planktonic spionid larvae are often the most common and abundant group in the coastal meroplankton (Anger et al. 1986; Levin 1986; Abe et al. 2011, 2014) because of their high abundance and species richness in coastal zones, high reproductive capacity, and relatively long planktonic stage (Blake 1996; Blake and Arnofsky 1999). As they are often seasonally dominant in coastal zooplankton communities, spionid larvae can play a major role in planktonic trophic dynamics (Martin et al. 1996; Pedersen et al. 2010). They are also reported to constitute a large portion of ballast water species (Carlton and Geller 1993; Carlton 1996). Spionidae includes species that adult inhabit a wide range of substrates and some are symbionts of other invertebrates (Martin and Britayev 1998, 2018; Sato-Okoshi 1999, 2000; Abe et al. 2019b). Among these, symbionts polydorids (i.e., from the *Polydora* complex or tribe Polydorini, see Radashevsky 2012) are well known as harmful pests in molluscan aquaculture because of their shell boring activities (Blake and Evans 1972; Handley and Bergquist 1997; Simon et al. 2006; Simon and Sato-Okoshi 2015). Understanding larval dynamics and dispersal is important to prevent the settlement of pest spionid species on the shells of aquaculture mollusks (Sato-Okoshi et al. 1990; Simon 2015; David et al. 2016). The host/substrate selectivity and settlement mechanism of spionid larvae during their developmental process are also interesting aspects of larval biology. Although various morphological characteristics of larvae including body shape, pigment patterns (placement and number), ciliary organization, and to some extent, chaetae can be generally used to identify the planktonic larvae of spionid species (Blake and Arnofsky 1999), species-level identification is still difficult because of the lack of information on the larval forms of many species.

The link between larval and adult form has been traditionally achieved by labor-intensive culturing approaches either through rearing larvae collected from plankton or by spawning adults in the laboratory (Shanks 2001). In recent years, ecological studies on the diversity and distribution of marine planktonic larvae are increasingly depending on molecular methods for accurate taxonomic identification to species level (Andre et al. 1999; Hosoi et al. 2004; Pradillon et al. 2007; Phillips et al. 2008; Heimeier et al. 2010). For future metabarcoding studies, establishment of a comprehensive DNA barcoding library is very useful for rapid identification of planktonic larvae. Meanwhile, the use of molecular methods for identifying planktonic larvae in extensive field surveys handling large numbers of collected samples still requires extensive cost. Since the direct microscopic observation, which allows prompt identification of larvae at low cost, remains the popular technique for distinguishing planktonic larvae, information on larval morphology would be useful for such studies.

The aim of the present study is identification of spionid larvae that dominantly appear among the planktonic polychaete larvae from northeastern Japan (Abe et al. 2011, 2014) by comparing adult and larval gene sequences. The 18S rRNA and 16S

rRNA genes was herein used as a marker for species-level discrimination in Spionidae. Moreover, we report the results of preliminary phylogenetic analysis using these genetic regions and describe the morphologies of spionid larvae with photomicrographs of living specimens.

## Materials and methods

### Sample collection and morphological observation

Planktonic larvae of spionid polychaetes were collected mainly from a coastal station in Onagawa Bay (38°26'15"N, 141°27'42"E; depth: 22 m), but also from Gobu-ura (38°24'01"N, 141°27'59"E), Sasuhama (38°24'22"N, 141°22'08"E), Sendai Port (38°16'22"N, 141°00'01"E), and Gamo Lagoon (38°15'18"N, 141°00'48"E) in Miyagi Prefecture, northeastern Japan, and Tomiura (35°02'20"N, 139°49'16"E) in Boso Peninsula and Habu Port (34°41'09"N, 139°26'16"E) in Izu-Oshima Island in eastern Japan (Table 1, Fig. 1). Plankton samples were collected in Onagawa Bay once a month, from April 2011 to August 2012, by vertical hauls from the bottom to the surface using a NORPAC net (Motoda 1957) with a mesh size of 110 μm. In the other areas, the plankton samples were collected in 2011–2016 by using a simple plankton net with a mesh size of 100 μm and a mouth diameter of 30 cm. Morphological characteristics of live spionid larvae were observed under stereomicroscopes (Leica, WILD MZ8; Olympus, SZX 16), and light photomicrographs were taken by using digital cameras (Nikon E950, E4500; Olympus DP25, DP73; Sony α6000) attached to the microscope. The larvae were anesthetized with magnesium chloride solution when necessary before the photography. Background, brightness, and contrast of the obtained images were adjusted using GNU Image Manipulation Program (GIMP) 2.10.6 (www.gimp.org). The terms trochophore, metatrochophore, and nectochaeta were defined as larvae with prototroch, clear signs of segmentation, and functional parapodia, respectively according to Rouse (2006).

Adult spionid polychaetes were collected from coastal waters in Shinminato (45°12'27"N, 141°08'09"E) and Numaura (45°06'54.0"N, 141°17'10.0"E) in Rishiri Island, Onagawa Bay, Sasuhama, Matsushima Bay (38°19'54"N, 141°08'44"E), Gamo Lagoon, Ninzaki (37°12'14"N, 136°55'07"E) and Kashima (37°05'13"N, 136°55'35"E) in Nanao Bay, Iwaki (36°55'14"N, 140°51'31"E), Moroiso Bay (35°09'27"N, 139°36'43"E), Ena Bay (35°08'46"N, 139°39'57"E), Tomiura, Akinohama (34°47'12"N, 139°24'32"E) in Izu-Oshima Island, Ishigaki Island (24°24'01"N, 124°08'30"E), and from a 103-m depth (by dredging) in Sagami Bay (35°05'N, 139°37'E) in Japan in 2011–2018 (Table 1, Fig. 1). Specimens were fixed in 70% or 99% ethanol. These fixed specimens of adult spionids were observed under stereomicroscopes (Leica, WILD MZ8; Olympus, SZX 16) and a biological light microscope (Nikon, Eclipse80i) and identified based on their mor-

**Table 1.** Spionid polychaete species collected from Japan in the present and previous studies. Data on life stage (adult/larva), type, and sampling locality, DDBJ/EMBL/GenBank accession numbers, and sequence lengths are given. Accession numbers of gene sequences newly obtained in the present study are highlighted in boldface type.

| Classification | Type locality | Sampling locality | | Accession no. (length: bp) | | | |
|---|---|---|---|---|---|---|---|
| | | | | 18S | | 16S | |
| | | Adult | Larvae | Adult | Larvae | Adult | Larvae |
| Nerininae Söderström, 1920 | | | | | | | |
| *Aonides* Claparède, 1864 | | | | | | | |
| *Aonides* aff. *oxycephala* (Sars, 1862) | Norway | Onagawa Bay | Onagawa Bay | **LC545853** (1753) | **LC545854** (1753) | **LC595683** (498) | **LC595684** (498) |
| *Laonice* Malmgren, 1867 | | | | | | | |
| *Laonice* sp. 1 | – | Onagawa Bay | Onagawa Bay | **LC545855** (1754) | **LC545856** (1754) | **LC595685** (504) | **LC595686** (504) |
| *Malacoceros* Quatrefages, 1843 | | | | | | | |
| *Malacoceros indicus* (Fauvel, 1928) | Gulf of Mannar | Ishigaki Island | – | **LC545857** (1757) | – | **LC595687** (510) | – |
| *Malacoceros* sp. | – | Iwaki | – | **LC545858** (1761) | – | **LC595688** (503) | – |
| *Paraprionospio* Caullery, 1914 | | | | | | | |
| *Paraprionospio coora* Wilson, 1990 | Australia | Onagawa Bay | Onagawa Bay, Sasuhama | **LC545859** (1754) | **LC545860** (1754) | **LC595689** (500) | **LC595690** (500) |
| *Paraprionospio patiens* Yokoyama, 2007 | Japan | Ena Bay | – | **LC545861** (1709) | – | **LC595691** (500) | – |
| *Poecilochaetus* Claparède in Ehlers, 1875 | | | | | | | |
| *Poecilochaetus* sp. | – | – | Onagawa Bay | – | **LC545862** (1765) | – | **LC595692** (509) |
| *Prionospio* Malmgren, 1867 | | | | | | | |
| *Prionospio* aff. *cirrifera* Wirén, 1883 | – | Sasuhama | – | **LC545863** (1752) | – | **LC595693** (497) | – |
| *Prionospio elongata* Imajima, 1990 | Japan | Nanao Bay | – | **LC545864** (1752) | – | **LC595694** (466) | – |
| *Prionospio japonica* Okuda, 1935 | Japan | Gamo Lgoon | – | **LC545865** (1730) | – | **LC595695** (509) | – |
| *Prionospio krusadensis* Fauvel, 1929 | Gulf of Manaar | Sasuhama | Onagawa Bay | **LC545866** (1749) | **LC545867** (1751) | **LC595696** (507) | **LC595697** (507) |
| *Prionospio lineata* Imajima, 1990 | Japan | Nanao Bay | – | **LC545868** (1752) | – | **LC595698** (506) | – |
| *Prionospio membranacea* Imajima, 1990 | Japan | Onagawa Bay | Onagawa Bay | **LC545869** (1752) | **LC545870** (1752), **LC545876** (1750) | **LC595699** (505) | **LC595700** (505), **LC595701** (505) |
| *Prionospio* cf. *saccifera* Mackie & Hartley, 1990 | Hong Kong | Onagawa Bay | – | **LC545871** (1753) | – | **LC595702** (497) | – |
| *Prionospio sexoculata* Augener, 1918 | Namibia | Onagawa Bay | – | **LC545872** (1752) | – | **LC595703** (459) | – |
| *Prionospio variegata* Imajima, 1990 | Japan | Akinohama | – | **LC545873** (1753) | – | **LC595704** (505) | – |
| *Prionospio* sp.1 | – | – | Onagawa Bay | – | **LC545874** (1752) | – | **LC595705** (500) |
| *Prionospio* sp.2 | – | – | Onagawa Bay | – | **LC545875** (1752) | – | **LC595706** (494) |
| *Rhynchospio* Hartman, 1936 | | | | | | | |
| *Rhynchospio* aff. *asiatica* sensu Radashevsky et al. (2014) | – | Gamo Lagoon, Sasuhama | Onagawa Bay, Gamo Lagoon, Sasuhama | **LC545877** (1783) | **LC545878** (1783) | **LC595707** (503) | **LC595708** (477) |
| *Scolelepis* Blainville, 1828 | | | | | | | |
| *Scolelepis* aff. *daphoinos* Zhou, Ji & Li, 2009 | China | Rishiri Island | | **LC545879** (1819) | | **LC595709** (505) | – |

| Classification | Type locality | Sampling locality | | Accession no. (length: bp) | | | |
|---|---|---|---|---|---|---|---|
| | | | | 18S | | 16S | |
| | | Adult | Larvae | Adult | Larvae | Adult | Larvae |
| *Scolelepis* cf. *kudenovi* Hartmann-Schröder, 1981 | Australia | | Sasuhama | | **LC545880** (1819) | – | **LC595710** (505) |
| *Scolelepis planata* Imajima, 1992 | Japan | Ena Bay | – | **LC545881** (1816) | – | **LC595711** (501) | – |
| *Scolelepis texana* Foster, 1971 | USA | Nanao Bay, Matsukawa–ura Lagoon | – | **LC545882** (1821) | – | **LC595712** (501) | – |
| *Scolelepis* sp. 1 | – | Onagawa Bay | Onagawa Bay | **LC545883** (1819) | **LC545884** (1819) | **LC595713** (505) | **LC595714** (505) |
| *Scolelepis* sp. 2 | – | – | Onagawa Bay | – | **LC545885** (1820) | – | **LC595715** (505) |
| *Spiophanes* Grube, 1860 | | | | | | | |
| *Spiophanes* aff. *kroyeri* Grube, 1860 | Greenland Sea | Onagawa Bay | – | **LC545886** (1750) | – | **LC595716** (500) | – |
| *Spiophanes uschakowi* Zachs, 1933 | Russia | – | Onagawa Bay | – | **LC545887** (1750) | – | **LC595717** (504) |
| *Spiophanes* aff. *uschakowi* Zachs, 1933 | Russia | Sasuhama | Onagawa Bay, Sasuhama | **LC545888** (1750) | **LC545889** (1750) | **LC595718** (504) | **LC595719** (504) |
| *Spiophanes wigleyi* Pettibone, 1962 | Georges Bank | Sagami Bay | – | **LC545890** (1749) | – | **LC595720** (513) | – |
| Spioninae Söderström, 1920 | | | | | | | |
| *Boccardia* Carazzi, 1893 | | | | | | | |
| *Boccardia proboscidea* Hartman, 1940 | USA | Sasuhama | Sasuhama | LC107607 (1768)[e] | **LC545891** (1768) | **LC595721** (472) | **LC595722** (472) |
| *Boccardia pseudonatrix* Day, 1961 | South Africa | Tomiura | Tomiura | **LC545892** (1745) | **LC545893** (1745) | **LC595723** (466) | **LC595724** (466) |
| *Boccardia* sp. 1 | – | – | Onagawa Bay | – | **LC545894** (1705) | – | **LC595725** (472) |
| *Boccardia* sp. 2 | – | – | Onagawa Bay, Sashama, Sendai Port | – | **LC545895** (1705) | – | **LC595726** (472) |
| *Boccardiella* Blake & Kudenov, 1978 | | | | | | | |
| *Boccardiella hamata* (Webster, 1879) | USA | Sasuhama, Gamo Lagoon | Onagawa Bay, Gobu–ura, Sasuhama | LC107608 (1772)[e] | **LC545896** (1772) | **LC595727** (472) | **LC595728** (472) |
| *Dipolydora* Verrill, 1881 | | | | | | | |
| *Dipolydora armata* (Langerhans, 1880) | Madeira | Akinohama | – | **LC545897** (1772) | – | **LC595729** (473) | – |
| *Dipolydora bidentata* (Zachs, 1933) | Russia | Sasuhama | Onagawa Bay | LC107609 (1770)[e] | **LC545898** (1770) | **LC595730** (475) | **LC595731** (475) |
| *Dipolydora* cf. *commensalis* (Andrews, 1891) | USA | – | Sasuhama | – | **LC545899** (1769) | – | **LC595732** (474) |
| *Dipolydora giardi* (Mesnil, 1893) | France | Onagawa Bay | Onagawa Bay | **LC545900** (1770) | **LC545901** (1766) | **LC595733** (474) | **LC595734** (474) |
| *Dipolydora* cf. *socialis* (Schmarda, 1861) | Chile | Onagawa Bay, Sasuhama | Onagawa Bay | **LC545902** (1770) | **LC545903** (1770) | **LC595735** (475) | **LC595736** (475) |
| *Dipolydora* sp. | – | – | Onagawa Bay | – | **LC545904** (1770) | – | **LC595737** (476) |
| *Polydora* Bosc, 1802 | | | | | | | |
| *Polydora aura* Sato-Okoshi, 1998 | Japan | Hiroshima Bay | – | AB705409 (1771)[a] | – | LC500931 (473)[g] | – |
| *Polydora brevipalpa* Zachs, 1933 | Russia | Mutsu Bay, Onagawa Bay | Onagawa Bay, Sasuhama | AB705407 (1771)[a] | **LC545905** (1766) | **LC595738** (474) | **LC595739** (474) |
| *Polydora calcarea* (Templeton, 1836) | UK | Kitaibaraki | – | AB705403 (1771)[b] | – | **LC595740** (475) | – |
| *Polydora cornuta* Bosc, 1802 | USA | Sasuhama, Gamo Lagoon | Gamo Lagoon | LC541483 (1742)[g] | **LC545906** (1770) | LC541484 (470)[g] | **LC595741** (470) |

| Classification | Type locality | Sampling locality | | Accession no. (length: bp) | | | |
|---|---|---|---|---|---|---|---|
| | | | | 18S | | 16S | |
| | | Adult Larvae | | Adult Larvae | | Adult Larvae | |
| *Polydora* cf. *glycymerica* Radashevsky, 1993 | Russia | – | Onagawa Bay, Sendai Port | – | **LC545907** (1771) | – | **LC595742** (472) |
| *Polydora hoplura* Claparède, 1868 | Italy | Kitaibaraki | Onagawa Bay, Gobu–ura | LC101841 (1771)[c] | **LC545908** (1769) | LC101870 (475)[c] | **LC595743** (475) |
| *Polydora neocaeca* Williams & Radashevsky, 1999 | USA | Hiroshima Bay | – | AB705404 (1771)[b] | – | **LC595744** (471) | – |
| *Polydora onagawaensis* Teramoto, Sato-Okoshi, Abe, Nishitani & Endo, 2013 | Japan | Onagawa Bay | Onagawa Bay | AB691768 (1771)[d] | **LC545909** (1771) | **LC595745** (473) | **LC595746** (473) |
| *Polydora* cf. *spongicola* Berkeley & Berkeley, 1950 | Canada | Moroiso Bay | Sasuhama | **LC545910** (1771) | **LC545911** (1771) | **LC595747** (475) | **LC595748** (475) |
| *Polydora websteri* Hartman in Loosanoff & Engle, 1943 | USA | Nakatsu tidal flats | – | AB705402 (1771)[b] | – | **LC595749** (468) | – |
| *Polydora* sp. 1 | – | Sasuhama | Onagawa Bay, Sasuhama | **LC545912** (1771) | **LC545913** (1771) | **LC595750** (476) | **LC595751** (476) |
| *Polydora* sp. 2 | – | – | Sasuhama, Gamo Lagoon | – | **LC545914** (1771) | – | **LC595752** (4702) |
| *Polydora* sp. 3 | – | – | Onagawa Bay, Sasuhama | – | **LC545915** (1771) | – | **LC595753** (473) |
| *Pseudopolydora* Czerniavsky, 1881 | | | | | | | |
| *Pseudopolydora* aff. *achaeta* Radashevsky & Hsieh, 2000 | Taiwan | Onagawa Bay | Onagawa Bay | LC019989 (1773)[e] | **LC545916** (1773) | **LC595754** (468) | **LC595755** (468) |
| *Pseudopolydora* cf. *kempi* (Southern, 1921) | India | Gamo Lagoon | Gamo Lagoon | LC019990 (1772)[e] | **LC545917** (1772) | **LC595756** (471) | **LC595757** (471) |
| *Pseudopolydora paucibranchiata* (Okuda, 1937) | Japan | Mangoku–ura Inlet | Onagawa Bay | LC019991 (1784)[e] | **LC545918** (1784) | **LC595758** (455) | **LC595759** (455) |
| *Pseudopolydora* cf. *reticulata* Radashevsky & Hsieh, 2000 | Taiwan | Gamo Lagoon | Onagawa Bay, Gamo Lagoon, Sendai Port | LC019988 (1775)[e] | **LC545919** (1775) | **LC595760** (470) | **LC595761** (470) |
| *Pseudopolydora tsubaki* Simon, Sato-Okoshi & Abe, 2017 | Japan | Habu Port | Tomiura, Habu Port | AB973929 (1713)[f] | **LC545920** (1749) | LC107857 (475)[f] | **LC595762** (425) |
| *Pseudopolydora ushioni* Simon, Sato-Okoshi & Abe, 2017 | Japan | Uranouchi Bay | – | AB973927 (1713)[f] | – | LC107855 (474)[f] | – |
| *Pseudopolydora* sp. | – | – | Sasuhama | – | **LC545921** (1781) | – | **LC595763** (471) |
| *Spio* Fabricius, 1785 | | | | | | | |
| *Spio* sp. 1 | – | Rishiri Island | Onagawa Bay | **LC545922** (1762) | **LC545923** (1762) | **LC595764** (467) | **LC595765** (467) |
| *Spio* sp. 2 | – | Sasuhama, Matsushima Bay | Onagawa Bay, Sasuhama | **LC545924** (1760) | **LC545925** (1760) | **LC595766** (462) | **LC595767** (462) |

[a]: Sato-Okoshi and Abe (2012); [b]: Sato-Okoshi and Abe (2013); [c]: Sato-Okoshi et al. (2017); [d]: Teramoto et al. (2013); [e]: Abe et al. (2016); [f]: Simon et al. (2019); [g]: Abe & Sato-Okoshi (2020).

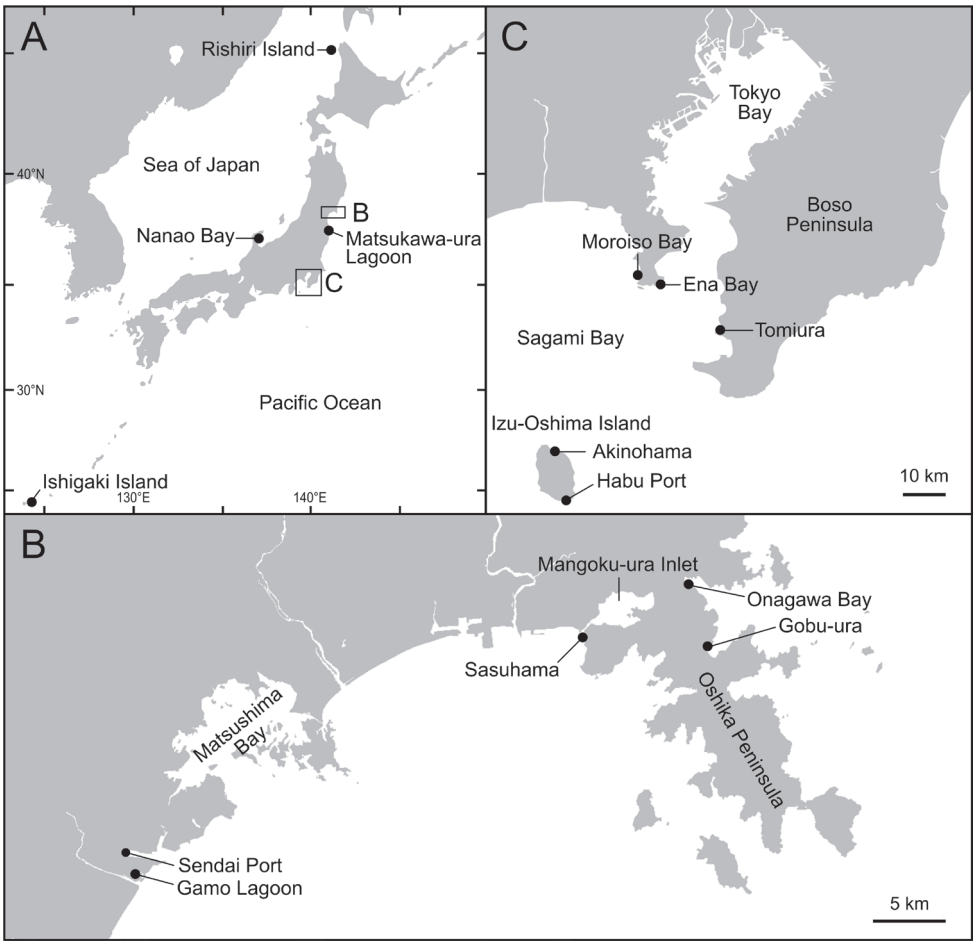


**Figure 1.** Maps showing sampling locations of the present study.

phological characteristics. Following Blake (2006) and Radashevsky et al. (2018), the genera *Poecilochaetus* and *Trochochaeta* were considered as belonging to the family Spionidae.

## DNA analysis and larval identification

Adults and larvae of one or more individuals, respectively, were subjected to DNA analysis. In order to clarify the development links between the different stages, we analyzed the DNA of as many larvae of different stages as possible. Except for *Laonice* sp. 2 (Fig. 4E), all larvae pictured in this paper have been identified by DNA analysis. All individuals were washed by several transfers in sterile filtered (pore size 0.2 μm) seawater and distilled water to remove as much extraneous matter as possible before

DNA extraction. Genomic DNA was extracted from live or ethanol-preserved larval (from the whole body) and adult (from palp or a small piece of tissue) spionid specimens by grinding and heating at 95 °C for 20 min in 50 µl TE buffer (pH 8.0) with 10% Chelex 100 (Bio-Rad; Richlen and Barber 2005). Undiluted or 10-fold diluted extracted DNA in TE buffer was used as template for polymerase chain reaction (PCR) depending on the DNA concentration. Partial sequences of nuclear 18S rRNA gene were amplified by PCR according to the methods described by Sato-Okoshi and Abe (2012, 2013) and Teramoto et al. (2013) using the following primer pairs (Nishitani et al. 2012): 18S-1F1 (AACCTGGTTKATCCTGCCAG) and 18S-1R632 (ACTACGAGCTTTTTAACYGCARC), 18S-2F576 (GGTAATTCCAGCTCYAATRG) and 18S-2R1209 (AAGTTTYCCCGTGTTGARTC), and 18S-3F1129 (GCTGAAACTTAAAGRAATTGACGG) and 18S-R1772 (TCACCTACGGAAACCTTGTTACG). Partial sequences of mitochondrial 16S rRNA gene were amplified by PCR according to the methods described by Abe et al. (2019a) using the 16Sar (CGCCTGTTTATCAAAAACAT) and 16Sbr (CCGGTCTGAACTCAGATCACGT) primer pair (Palumbi et al. 1991). The PCR products were purified using ExoSAP-IT (Affymetrix, Cleveland, OH, USA) 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, USA). Larval and adult gene sequences obtained in the present study (Table 1) were aligned using the MAFFT online service ver. 7 with the L-INS-i algorithm (Katoh et al. 2017) with (Fig. 2) and without (Fig. 3) the sequences of other spionid species available in the DNA Data Bank of Japan (DDBJ), the European Nucleotide Archive (ENA), or GenBank databases (Table 2). The 18S and 16S ribosomal RNA gene sequences of *Sabella pavonina* Savigny, 1822 (DDBJ/EMBL/GenBank ID: U67144 and AY340482) and *Laonome* sp. (KP793139 and KP793138) obtained from DDBJ/ENA/GenBank were used as outgroup taxa. Ambiguously aligned regions of 2 alignments were eliminated by employing Gblocks (Talavera and Castresana 2007) implemented in PhyloSuite v.1.2.2 (Zhang et al. 2020) with the following relaxed settings: minimum number of sequences for a conserved/flank position: half the number of sequences + 1, maximum number of contiguous non-conserved positions: 10, minimum length of a block: 5, and with half of the allowed gap positions. The final lengths of the alignments were 1738 (18S) and 447 (16S) bp for the multiple sequence alignment (MSA) without DDBJ/ENA/GenBank sequences and 1644 (18S) and 410 (16S) bp for MSA with DDBJ/ENA/GenBank sequences. Phylogenetic trees were constructed based on the concatenated sequences of 18S and 16S rRNA gene region by maximum likelihood (ML) analyses performed using IQ-TREE (Nguyen et al. 2015) implemented in PhyloSuite under Edge-linked partition model. The TIM2e+I+G4 and TIM2+F+I+G4 models were selected for the 18S and 16S rRNA gene region, respectively as the best substitution model by ModelFinder (Kalyaanamoorthy et al. 2017) as implemented in IQ-TREE under the Bayesian information criterion (BIC). The robustness of the ML trees was evaluated by the Shimodaira–Hasegawa–like approximate likelihood-ratio test (SH-

**Table 2.** Terminal taxa whose sequences were obtained from DDBJ/EMBL/GenBank and herein used in the phylogenetic analyses. Type and collection localities, accession numbers, sequence lengths, and references are shown.

| Classification | Type locality | Collection locality | Accession number (Length: bp) | | Reference |
|---|---|---|---|---|---|
| | | | 18S | 16S | |
| Spionidae | | | | | |
| Nerininae Söderström, 1920 | | | | | |
| *Aonidella* López-Jamar, 1989 | | | | | |
| *Aonidella* cf. *dayi* Maciolek in López-Jamar, 1989 | Gulf of Cadiz, Spain | Great Meteor Seamount, NE Atlantic | KF434504 (483) | KF434508 (443) | Meißner et al. (2014) |
| *Aonides* Claparède, 1864 | | | | | |
| *Aonides oxycephala* (Sars, 1862) | Norway | France | MG913226 (1699) | MG878895 (337) | Radashevsky et al. (unpubl.) |
| *Aonides selvagensis* Brito, Núñez & Riera, 2006 | Savage Islands, Portugal | Irving Seamount, NE Atlantic | KF434507 (516) | – | Meißner et al. (2014) |
| *Aurospio* Maciolek, 1981 | | | | | |
| *Aurospio dibranchiata* Maciolek, 1981 | Argentine Basin, SW Atlantic | Kaplan, Pacific Mn nodule province | EU340091 (1797) | EU340087 (484) | Mincks et al. (2009) |
| *Aurospio foodbancsia* Mincks, Dyal, Paterson, Smith & Glover, 2009 | Bellingshausen Sea, Antarctica | West Antarctic Peninsula shelf | EU340097 (1765) | EU340078 (552) | Mincks et al. (2009) |
| *Aurospio* sp. Q | – | India | – | KF459948 (443) | Periasamy et al. (unpubl.) |
| *Aurospio* sp. R | – | Ross Sea | – | KF713473 (397) | Gallego et al. (2014) |
| *Aurospio* sp. S | – | Eastern Vema Fracture Zone | MN447187 (844) | MN441726 (409) | Guggolz et al. (2020) |
| *Aurospio* sp. T | – | Clarion Clipperton Fracture Zone | – | MN441512 (411) | Guggolz et al. (2020) |
| *Dispio* Hartman, 1951 | | | | | |
| *Dispio remanei* Friedrich, 1956 | Pacific Ocean, Central America | Brazil | KU900474 (671) | – | Rebelo & Schettini (unpubl.) |
| *Glandulospio* Meißner, Bick, Guggolz & Götting, 2014 | | | | | |
| *Glandulospio orestes* Meißner, Bick, Guggolz & Götting, 2014 | Little Meteor Seamount, NE Atlantic | Little Meteor Seamount, NE Atlantic | KF434505 (402) | KF434511 (446) | Meißner et al. (2014) |
| *Laonice* Malmgren, 1867 | | | | | |
| *Laonice* cf. *antarctica* Hartman, 1953 | Rio Grande do Sul | Antarctic | | KX867280 (373) | Brasier et al. (2016) |
| *Laonice cirrata* (M. Sars, 1851) | Norway | Russia | KM998754 (1744) | – | Radashevsky et al. (unpubl.) |
| *Laonice norgensis* Sikorski, 2003 | Norwegian Sea, North Atlantic | Little Meteor Seamount | KF434506 (514) | KF434512 (454) | Meißner et al. (2014) |
| *Laonice* cf. *vieitezi* López, 2011 | Bellingshausen Sea, West Antarctica | Antarctic | – | KX867288 (368) | Brasier et al. (2016) |
| *Laonice weddellia* Hartman, 1978 | Weddell Sea | Antarctic | – | KX867313 (379) | Brasier et al. (2016) |
| *Lapnice* sp. VR-2006 | – | Bohuslän, Sweden | DQ779655 (1705) | DQ779619 (342) | Rousset et al. (2007) |
| *Laonice* sp. SLM-2008 | – | California borderland basins, USA | EU340089 (1784) | EU340088 (546) | Mincks et al. (2009) |
| *Laonice* sp. A | – | Eastern Vema Fracture Zone | MK507647 (1017) | MK507653 (469) | Guggolz et al. (2019) |
| *Laonice* sp. B | – | Eastern Vema Fracture Zone | MK507651 (1017) | MK507657 (470) | Guggolz et al. (2019) |
| *Laonice* sp. C | – | Western Vema–Fracture Zone | MK507650 (1017) | MK507658 (450) | Guggolz et al. (2019) |
| *Laonice* sp. D | – | Western Vema–Fracture Zone | MK507638 (1017) | MK507723 (475) | Guggolz et al. (2019) |
| *Laonice* sp. E | – | Western Vema–Fracture Zone | MK507644 (966) | MK507706 (473) | Guggolz et al. (2019) |

| Classification | Type locality | Collection locality | Accession number (Length: bp) | | Reference |
|---|---|---|---|---|---|
| | | | 18S | 16S | |
| *Laonice* sp. F | – | Vema Transform Fault | MK507623 (1017) | MK507718 (473) | Guggolz et al. (2019) |
| *Laonice* sp. G | – | Puerto Rico Trench | MK507624 (1017) | MK507708 (474) | Guggolz et al. (2019) |
| *Laonice* sp. H | – | Puerto Rico Trench | MK507617 (1017) | MK507720 (474) | Guggolz et al. (2019) |
| *Malacoceros* Quatrefages, 1843 | | | | | |
| *Malacoceros fuliginosus* (Claparède, 1868) | Italy | St. Efflau, France | AY525632 (1765) | – | Struck and Purschke (2005) |
| | | Helgoland, Germany | – | EF431961 (417) | Blank and Bastrop (2009) |
| *Malacoceros indicus* (Fauvel, 1928) | Gulf of Mannar | Lizard Island, Australia | KP636512 (454) | KP636511 (391) | Meißner and Götting (2015) |
| *Marenzelleria* Mesnil, 1896 | | | | | |
| *Marenzelleria arctia* (Chamberlin, 1920) | Beaufort Sea | Kara Sea, Russia | KJ546264 (1775) | KJ546306 (343) | Radashevsky et al. (2014) |
| *Marenzelleria bastropi* Bick, 2005 | North Carolina,USA | USA | EF446959 (468), EF446967 (577) | EF431963 (419) | Blank and Bastrop (2009) |
| *Marenzelleria neglecta* Sikorski & Bick, 2004 | Germany | Baltic Sea | EF446955 (470), EF446963 (578) | DQ309248 (419) | Bastrop and Blank (2006), Blank and Bastrop (2009) |
| *Marenzelleria viridis* (Verrill, 1873) | New Jersey, USA | Barlow's Landing, MA, USA | EU418860 (1810) | – | Struck et al. (2008) |
| | | Ringkøbing Fjord | – | DQ309252 (419) | Bastrop and Blank (2006) |
| *Marenzelleria wireni* Augener, 1913 | Franz Jozef Land, Russia | Spitsbergen, Norway | EF446957 (472), EF446965 (579) | EF431980 (417) | Blank and Bastrop (2009) |
| *Paraprionospio* Caullery, 1914 | | | | | |
| *Paraprionospio cordifolia* Yokoyama, 2007 | Wakasa Bay, Japan | Eastern Arabian Sea, India | KT900309 (1655) | – | Rengaiyan and Ingole (2018) |
| *Paraprionospio cristata* Zhou,Yokoyama & Li, 2008 | East China Sea, China | India | KY704338 (520) | – | Vijapure et al. (unpubl.) |
| *Paraprionospio patiens* Yokoyama, 2007 | Osaka Bay, Japan | India | KT900307 (1684) | KY704331 (519) | Rengaiyan and Ingole (2018), Vijapure et al. (unpubl.) |
| *Paraprionospio* sp. EPK-2019 | – | – | MN069511 (588) | – | Kiskaddon et al. (unpubl.) |
| *Poecilochaetus* Claparède in Ehlers, 1875 | | | | | |
| *Poecilochaetus serpens* Allen, 1904 | English Channel | Arcachon, France | AY569652 (1833) | AY569680 (463) | Bleidorn et al. (2005) |
| *Poecilochaetus* sp. VR-2006 | – | Banyuls, France | DQ779667 (1710) | DQ779630 (344) | Rousset et al. (2007) |
| *Poecilochaetus* sp. 18 PB | – | Clarion–Clipperton Fracture Zone | – | MK971106 (419) | Bonifácio et al. (2020) |
| *Prionospio* Malmgren, 1867 | | | | | |
| *Prionospio dubia* Day, 1961 | South Africa | Southern New England, MA, USA | EU418859 (1823) | – | Struck et al. (2008) |
| *Prionospio* sp. A | – | Clarion Clipperton Fracture Zone | – | MN441557 (416) | Guggolz et al. (2020) |
| *Prionospio* sp. B | – | Eastern Vema Fracture Zone | MN447146 (846) | MN441645 (331) | Guggolz et al. (2020) |
| *Prionospio* sp. C (as *Prionospio* sp. 29 PB) | – | Clarion–Clipperton Fracture Zone | MK971148 (1677) | MK971035 (422) | Bonifácio et al. (2020) |
| *Prionospio* sp. D | – | Eastern Vema Fracture Zone | MN447192 (842) | MN441641 (409) | Guggolz et al. (2020) |
| *Prionospio* sp. E (as *Prionospio ehlersi*) | – | CROZEX | EU340095 (1812) | EU340081 (549) | Mincks et al. (2009) |
| *Prionospio* sp. F | – | Clarion Clipperton Fracture Zone | – | MN441542 (405) | Guggolz et al. (2020) |
| *Prionospio* sp. G | – | Eastern Vema Fracture Zone | MN447188 (844) | MN441564 (397) | Guggolz et al. (2020) |
| *Prionospio* sp. H | – | Clarion Clipperton Fracture Zone/ eastern Vema Fracture Zone | MN447158 (844) | MN441554 (411) | Guggolz et al. (2020) |

| Classification | Type locality | Collection locality | Accession number (Length: bp) | | Reference |
|---|---|---|---|---|---|
| | | | 18S | 16S | |
| *Prionospio* sp. I | – | Puerto Rico Trench | MN447157 (844) | MN441749 (409) | Guggolz et al. (2020) |
| *Prionospio* sp. K | – | Clarion Clipperton Fracture Zone | – | MN441555 (413) | Guggolz et al. (2020) |
| *Prionospio* sp. L | – | Western Vema Fracture Zone | MN447168 (844) | MN441745 (408) | Guggolz et al. (2020) |
| *Prionospio* sp. M | – | Eastern Vema Fracture Zone | MN447160 (844) | MN441561 (411) | Guggolz et al. (2020) |
| *Prionospio* sp. N | – | Eastern Vema Fracture Zone | MN447180 (844) | MN441604 (342) | Guggolz et al. (2020) |
| *Prionospio* sp. O | – | Eastern Vema Fracture Zone | MN447159 (844) | MN441748 (342) | Guggolz et al. (2020) |
| *Prionospio* sp. P | – | Eastern Vema Fracture Zone | MN447173 (844) | MN441753 (339) | Guggolz et al. (2020) |
| *Prionospio* sp. KJO-2005 | – | Monterey Bay, CA, USA | DQ209226 (1703) | – | Osborn et al. (2007) |
| *Pygospio* Claparède, 1863 | | | | | |
| *Pygospio elegans* Claparède, 1863 | Normandy, France | Russia | KJ747074 (1719) | KJ747084 (468) | Radashevsky et al. (2016b) |
| *Pygospio* sp. 1 (as *Pygospio* sp. 2583) | – | Russia | KP940584 (1709) | KP940582 (306) | Radashevsky et al. (2016b) |
| *Pygospio* sp. 2 (as *Pygospio* sp. VVP-2014) | – | USA | KJ747077 (1756) | KJ747087 (306) | Radashevsky et al. (2016b) |
| *Rhynchospio* Hartman, 1936 | | | | | |
| *Rhynchospio arenicola* Hartman, 1936 | CA, USA | USA | KJ546286 (1737) | KJ546318 (341) | Radashevsky et al. (2014) |
| *Rhynchospio* aff. *asiatica* sensu Radashevsky et al. (2014) | – | South Korea | KJ546296 (1731) | KJ546345 (492) | Radashevsky et al. (2014) |
| *Rhynchospio darwini* Radashevsky, 2015 (as *Rhynchospio* sp. 44) | Australia | Australia | KP986493 (1789) | KP986492 (316) | Radashevsky et al. (2016a) |
| *Rhynchospio* cf. *foliosa* Imajima, 1991 (as *Rhynchospio foliosa*) | Japan | USA | KP986489 (1765) | KP986488 (450) | Radashevsky et al. (2016a) |
| *Rhynchospio glutaea* (Ehlers, 1897) | Strait of Magellan, Chile | Argentina | KJ546281 (1747) | KJ546332 (341) | Radashevsky et al. (2014) |
| *Rhynchospio mzansi* Simon, Williams & Henninger, 2018 | South Africa | South Africa | MF625258 (1662) | MF625254 (290) | Simon et al. (2019b) |
| *Rhynchospio nhatrangi* Radashevsky, 2007 | Vietnam | Vietnam | KJ546299 (1717) | KJ546343 (499) | Radashevsky et al. (2014) |
| *Scolelepis* Blainville, 1828 | | | | | |
| *Scolelepis acuta* (Treadwell, 1914) | San Diego, USA | Brazil | KU900479 (683) | – | Rebelo & Schettini (unpubl.) |
| *Scolelepis bonnieri* Mesnil, 1896 | English Chanel | Helgoland, Germany | EU084878 (1711) | – | Vortsepneva et al. (2008) |
| *Scolelepis chilensis* (Hartmann-Schröder, 1962) | Chile | Brazil | KU900475 (689) | – | Rebelo & Schettini (unpubl.) |
| *Scolelepis daphoinos* Zhou, Ji & Li, 2009 | China | China | – | GU362676 (461) | Zhou et al. (2010) |
| *Scolelepis eltaninae* Blake, 1983 | Ross Sea | Antarctica | KF713431 (333) | KF713470 (398) | Gallego et al. (2014) |
| *Scolelepis goodbodyi* (Jones, 1962) | Jamaica | Brazil | KU900477 (441) | – | Rebelo & Schettini (unpubl.) |
| *Scolelepis kudenovi* Hartmann-Schröder, 1981 | Australia | Lizard Island, Australia | KP636517 (464) | – | Meißner and Götting (2015) |
| *Scolelepis laonicola* (Tzetlin, 1985) (as *Asetocalamyzas laonicola*) | White Sea, Russia | White Sea, Russia | EF569206 (1323) | – | Vortsepneva et al. (2008) |
| *Scolelepis squamata* (Müller, 1806) | Denmark | Sylt, Germany | AF448164 (1848) | – | Bleidorn et al. (2003) |
| *Scolelepis* sp. sco206 | – | Eastern Arabian Sea, India | KT900310 (1759) | – | Rengaiyan and Ingole (2018) |
| *Scolelepis* sp. sco207 | – | Eastern Arabian Sea, India | KT900311 (1759) | – | Rengaiyan and Ingole (2018) |
| *Spiophanes* Grube, 1860 | | | | | |
| *Spiophanes berkeleyorum* Pettibone, 1962 | Vancouver Island, Canada | California, USA | MN186816 (1724) | – | Radashevsky et al. (2020a) |
| *Spiophanes bombyx* (Claparède, 1870) | Gulf of Naples, Italy | Adriatic Sea, Italy | – | MG878899 (484) | Radashevsky et al. (2020a) |
| *Spiophanes* cf. *convexus* Delgado-Blas, Díaz-Díaz & Viéitez, 2019 | Ria de Vigo, Spain | Brittany, France | MG913229 (1742) | MG878902 (505) | Radashevsky et al. (2020a) |

| Classification | Type locality | Collection locality | Accession number (Length: bp) | | Reference |
|---|---|---|---|---|---|
| | | | 18S | 16S | |
| *Spiophanes duplex* (Chamberlin, 1919) (as *Spiophanes berkeleyorum* isolate 20548.2) | California,USA | California,USA | MN186817 (1682) | – | Radashevsky et al. (2020a) |
| *Spiophanes hakaiensis* Radashevsky & Pankova in Radashevsky et al. 2020 | BritishColumbia, Canada | California, USA | MG913241 (1746) | MG878914 (369) | Radashevsky et al. (2020a) |
| *Spiophanes* cf. *kroyeri* Grube, 1860 | Greenland Sea, NW Atlantic | BarentsSea,Norway | MG913238 (1738) | MG878907 (340) | Radashevsky et al. (2020a) |
| *Spiophanes* aff. *kroyeri* Grube, 1860 (as *Spiophanes kroeyeri*) | Greenland Sea, NW Atlantic | | EU340094 (1769) | EU340080 (544) | Mincks et al. (2009) |
| *Spiophanes norrisi* Meißner & Blank, 2009 | Mexico | USA | GQ202716 (535) | – | Meißner and Blank (2009) |
| *Spiophanes pisinnus* Meißner & Hutchings, 2003 | New South Wales, Australia | Australia | GQ202721 (534) | – | Meißner and Blank (2009) |
| *Spiophanes soederstromi* Hartman, 1953 | off Rio Grande do Sul, Brazil | Paraná, Brazil | MG913232 (1735) | MG878905 (340) | Radashevsky et al. (2020a) |
| *Spiophanes uschakowi* Zachs, 1933 | northern Sea of Japan, Russia | Russia | KM998760 (1747) | MG878915 (342) | Radashevsky et al. (2020a) |
| *Spiophanes viriosus* Meißner & Hutchings, 2003 | Australia | Lizard Island, Australia | KP636519 (451) | – | Meißner and Götting (2015) |
| *Spiophanes* sp. A | – | East China Sea, South Korea | MG913244 (1732) | MG878920 (417) | Radashevsky et al. (2020a) |
| *Spiophanes* sp. RG-2014 | – | Antarctica | KF713435 (318) | KF713474 (372) | Gallego et al. (2014) |
| *Streblospio* Webster, 1879 | | | | | |
| *Streblospio benedicti* Webster, 1879 | New Jersey, USA | Netherlands | KC686673 (411) | – | van Pelt-Heerschap (unpubl.) |
| *Streblospio* sp. | – | India | KY704336 (578) | KY704328(523) | Vijapure et al. (unpubl.) |
| *Trochochaeta* Levinsen, 1884 | | | | | |
| *Trochochaeta multisetosa* (Örsted, 1844) | Danmark | Askeröfjord, Sweden/North Sea, Norway | MN296517 (1728) | MN193552 (341) | Radashevsky et al. (2020a) |
| Spioninae Söderström, 1920 | | | | | |
| *Boccardia* Carazzi, 1893 | | | | | |
| *Boccardia perata* (Chlebovitsch, 1959) | Kurile Islands | Sea of Japan, Russia | – | MH493047 (473) | Radashevsky et al. (2019) |
| *Boccardia polybranchia* (Haswell, 1885) | New South Wales, Australia | South Africa | KY677891 (1714) | – | Williams et al. (2017) |
| *Boccardia proboscidea* Hartman, 1940 | CA, USA | CA, USA | KJ546254 (1763) | MH493027 (435) | Radashevsky et al. (2014, 2019) |
| *Boccardia pseudonatrix* Day, 1961 | Knysna Estuary, South Africa | South Africa | KY677895 (1719) | – | Williams et al. (2017) |
| *Boccardiella* Blake & Kudenov, 1978 | | | | | |
| *Boccardiella hamata* (Webster, 1879) | USA | Incheon, South Korea | MT482710 (1741) | – | Lee et al. (2020) |
| *Dipolydora* Verrill, 1881 | | | | | |
| *Dipolydora bidentata* (Zachs, 1933) | northern Sea of Japan, Russia | Peter the Great Bay, Russia | JX228065 (900) | JX228103 (475) | Radashevsky and Pankova (2013) |
| *Dipolydora capensis* (Day, 1955) | South Africa | South Africa | KY677896 (1714) | – | Williams et al. (2017) |
| | | South Africa | KY677897 (1714) | – | Williams et al. (2017) |
| *Dipolydora cardalia* (E. Berkeley, 1927) | British Columbia, Canada | Peter the Great Bay, Sea of Japan | JX228073 (900) | JX228113 (475) | Radashevsky and Pankova (2013) |
| *Dipolydora carunculata* (Radashevsky, 1993) | Vostok Bay, Russia | Peter the Great Bay, Sea of Japan | JN048711 (942) | JN048698 (475) | Radashevsky and Pankova (2013) |
| *Dipolydora quadrilobata* (Jacobi, 1883) | Kiel Canal, Germany | Russia | – | MH493041 (309) | Radashevsky et al. (unpubl.) |

| Classification | Type locality | Collection locality | Accession number (Length: bp) | | Reference |
|---|---|---|---|---|---|
| | | | 18S | 16S | |
| *Dipolydora* cf. *socialis* (Schmarda, 1861) | Chile | South Africa | KY677899 (1715) | – | Williams et al. (2017) |
| *Microspio* Mesnil, 1896 | | | | | |
| *Microspio granulata* Blake & Kudenov, 1978 | Australia | Lizard Island, Australia | KP636515 (457) | KP636514 (362) | Meißner and Götting (2015) |
| *Polydora* Bosc, 1802 | | | | | |
| *Polydora brevipalpa* Zachs, 1933 | northern Sea of Japan | China | KP231289 (1725) | – | Ye et al. (2019) |
| *Polydora cornuta* Bosc, 1802 | South Carolina | Netherlands | KC686637 (421) | – | van Pelt-Heerschap (unpubl.) |
| *Polydora neocaeca* Williams & Radashevsky, 1999 (as *Polydora haswelli*) | Rhode Island | China | KF562242 (1792) | KF562235 (511) | Ye et al. (2019) |
| *Polydora lingshuiensis* Ye, Tang, Wu, Su, Wang, Yu & Wang, 2015 | China | China | KF562240 (1791) | KF562233 (462) | Ye et al. (2015) |
| *Polydora* cf. *nuchalis* Woodwick, 1953 | California, USA | South Africa | KY677903 (1715) | – | Williams et al. (2017) |
| *Polydora triglanda* Radashevsky & Hsieh, 2000 | Taiwan | Taiwan | JN048718 (941) | JN048705 (475) | Radashevsky and Pankova (2013) |
| *Polydora* cf. *websteri* Hartman in Loosanoff & Engle, 1943 | Milford Harbor, USA | South Africa | KY677904 (1716) | – | Williams et al. (2017) |
| *Polydorella* Augener, 1914 | | | | | |
| *Polydorella dawydoffi* Radashevsky, 1996 | South China Sea | Nha Trang Bay, Vietnam | – | MG460900 (308) | Radashevsky et al. (2020b) |
| *Pseudopolydora* Czerniavsky, 1881 | | | | | |
| *Pseudopolydora achaeta* Radashevsky & Hsieh, 2000 | Taiwan | Erhjen River, Tainan, Taiwan | – | MG460903 (304) | Radashevsky et al. (2020b) |
| *Pseudopolydora bassarginensis* (Zachs, 1933) | northern Sea of Japan | Vostok Bay, Sea of Japan, Russia | – | MG460894 (306) | Radashevsky et al. (2020b) |
| *Pseudopolydora dayii* Simon, 2009 | South Africa | South Africa | KY677907 (1716) | | Williams et al. (2017) |
| *Pseudopolydora diopatra* Hsieh, 1992 | Taiwan | Hsinchu, Taiwan | – | MG460906 (308) | Radashevsky et al. (2020b) |
| *Pseudopolydora eriyali* Simon, Sato-Okoshi & Abe, 2017 | South Africa | South Africa | AB973933 (1713) | LC107863 (471) | Simon et al. (2019) |
| *Pseudopolydora kempi japonica* Imajima & Hartman, 1964 | Japan | Vostok Bay, Sea of Japan, Russia | – | MG460897 (306) | Radashevsky et al. (2020b) |
| *Pseudopolydora paucibranchiata* (Okuda, 1937) | Japan | Gulf of Naples, Italy | – | MG460937 (455) | Radashevsky et al. (2020b) |
| *Pseudopolydora pulchra* (Carazzi, 1893) | Gulf of Naples, Mediterranean | Bay of Morlaix, Brittany, France | – | MG460932 (471) | Radashevsky et al. (2020b) |
| *Pseudopolydora uphondo* Simon, Sato-Okoshi & Abe, 2017 | South Africa | South Africa | LC107848 (1711) | LC107866 (472) | Simon et al. (2019) |
| *Pseudopolydora vexillosa* Radashevsky & Hsieh, 2000 (as *Pseudopolydora* sp. B) | Taiwan | Mung Is., Nha Trang Bay, Vietnam | – | MG460890 (295) | Radashevsky et al. (2020b) |
| *Pseudopolydora* sp. A | – | Northern Territory, Australia | – | MG460921 (296) | Radashevsky et al. (2020b) |
| *Pseudopolydora* sp. B (as *Pseudopolydora* sp. C) | – | Arabian Gulf, Kuwait | – | MG460957 (295) | Radashevsky et al. (2020b) |
| *Pseudopolydora* sp. C (as *Pseudopolydora* sp. D) | – | Arabian Gulf, Kuwait | – | MG460941 (309) | Radashevsky et al. (2020b) |
| *Pseudopolydora* sp. D (as *Pseudopolydora* sp. E) | – | Raunefjord, North Sea, Norway | – | MG460960 (305) | Radashevsky et al. (2020b) |
| *Pseudopolydora* sp. Sodwana 32-4 | – | South Africa | LC107849 (1724) | LC107867 (473) | Simon et al. (2019) |
| *Spio* Fabricius, 1785 | | | | | |
| *Spio arndti* Meißner, Bick & Bastrop, 2011 (as *Spio* sp. LK-2011-2) | Baltic Sea | Baltic Sea | FR823434 (1765) | FR823439 (453) | Meißner et al. (2011) |
| *Spio blakei* Maciolek, 1990 | Botany Bay, New South Wales, Australia | Lizard Island, Australia | KP636507 (458) | KP636502 (348) | Meißner and Götting (2015) |
| *Spio filicornis* (O. F. Müller, 1776) | Iluilårssuk, Greenland | Iluilårssuk, Greenland | FR823431 (1765) | FR823436 (454) | Meißner et al. (2011) |
| *Spio symphyta* Meißner, Bick & Bastrop, 2011 (as *Spio* sp. LK-2011-1) | North Sea | North Sea | FR823433 (1766) | FR823438 (453) | Meißner et al. (2011) |

| Classification | Type locality | Collection locality | Accession number (Length: bp) | | Reference |
|---|---|---|---|---|---|
| | | | 18S | 16S | |
| *Spio* sp. 2573 | – | Koni Peninsula, Sea of Okhotsk, Russia | KT200135 (1688) | KT200126 (310) | Radashevsky et al. (2016b) |
| Outgroup | | | | | |
| Sabellidae Latreille, 1825 | | | | | |
| *Laonome* Malmgren, 1866 | | | | | |
| *Laonome* sp. | – | Pärnu Bay, Baltic Sea | KP793139 (1813) | KP793138 (450) | Kotta et al. (2015) |
| *Sabella* Linnaeus, 1767 | | | | | |
| *Sabella pavonina* Savigny, 1822 | Plymouth | –/Brittany, France | U67144 (1726) | AY340482 (476) | Nadot & Grant (unpubl.), Rousset et al. (2007) |

aLRT) with 5,000 replicates (Guindon et al. 2010), approximate Bayes (aBayes) test (Anisimova et al. 2011), and ultrafast bootstraps (UFBoot) with 5000 replicates (Hoang et al. 2018). SH-aLRT ≥ 80%, aBayes ≥ 0.95, and UFBoot ≥ 95% were defined as robust statistical support. All the sequences newly generated in this study were deposited in the DDBJ/ENA/GenBank nucleotide sequence database under accession numbers LC545853 to LC545925 and LC595683 to LC595767 (Table 1). Part of the sequences used in the present study was reported in the previous studies (see Table 1). The planktonic spionid larvae were identified by comparing larval and adult sequences obtained in the present study and/or by larval morphology.

## Results

### The 18S and 16S rRNA gene analyses of larval and adult spionids

Nuclear 18S and mitochondrial 16S rRNA gene sequences of adult spionid polychaetes were successfully obtained from 48 species belonging to 14 genera (Table 1).

In the phylogenetic analysis using only the sequences obtained in the present study (i.e., without DBJ/ENA/GenBank sequences), species from the genera *Paraprionospio*, *Pseudopolydora*, *Scolelepis*, *Spio*, and *Spiophanes* were recovered as monophyletic groups with robust statistical supports (i.e., SH-aLRT ≥ 80%, aBayes ≥ 0.95, and UFBoot ≥ 95%, Fig. 2). Species belonging to the genus *Boccardia* except for *B. pseudonatrix* and those from the genus *Dipolydora* except for *D. armata* and *D.* cf. *commensalis* were recovered as monophyletic groups with robust statistical supports. The tribe Polydorini and subfamily Spioninae were also recovered as monophyletic although UFBoot of monophyly of the subfamily Spioninae was with low support (≤ 95%).

In the phylogenetic analysis with the sequences obtained in the present study and from DDBJ/ENA/GenBank databases, species belonging to the genera *Poecilochaetus*, *Laonice*, *Marenzelleria*, *Pseudopolydora*, *Pygospio*, *Rhynchospio*, *Scolelepis*, *Spio* + *Microspio*, and *Spiophanes*, were recovered as monophyletic groups with robust statistical supports (Fig. 3). Species belonging to the genus *Polydora* were recovered as a monophyletic group but with low UFBoot support. Tribe Polydorini + *Pygospio*, that plus *Glandulospio*, and *Spiophanes* + *Trochochaeta* were also recovered as monophyletic groups with robust statistical supports. The genera *Poecilochaetus* and *Trochochaeta*, which were previously considered as belonging to the family Poecilochaetidae and Trochochaetidae, respectively, were recovered as ingroup taxa of the family Spionidae with robust statistical supports (Fig. 3).

In total, 41 species belonging to 14 genera of planktonic spionid larvae were identified (Table 1; Fig. 2), 27 of which were identified by the 100% or nearly 100% match between the sequences obtained from adult and larvae (Fig. 2). The other 14 species of spionid larvae were identified to species or genus level based on their phylogenetic position and/or larval morphology. Tentative larval diagnosis for each genus and larval identification keys to species of each genus based on the morphol-

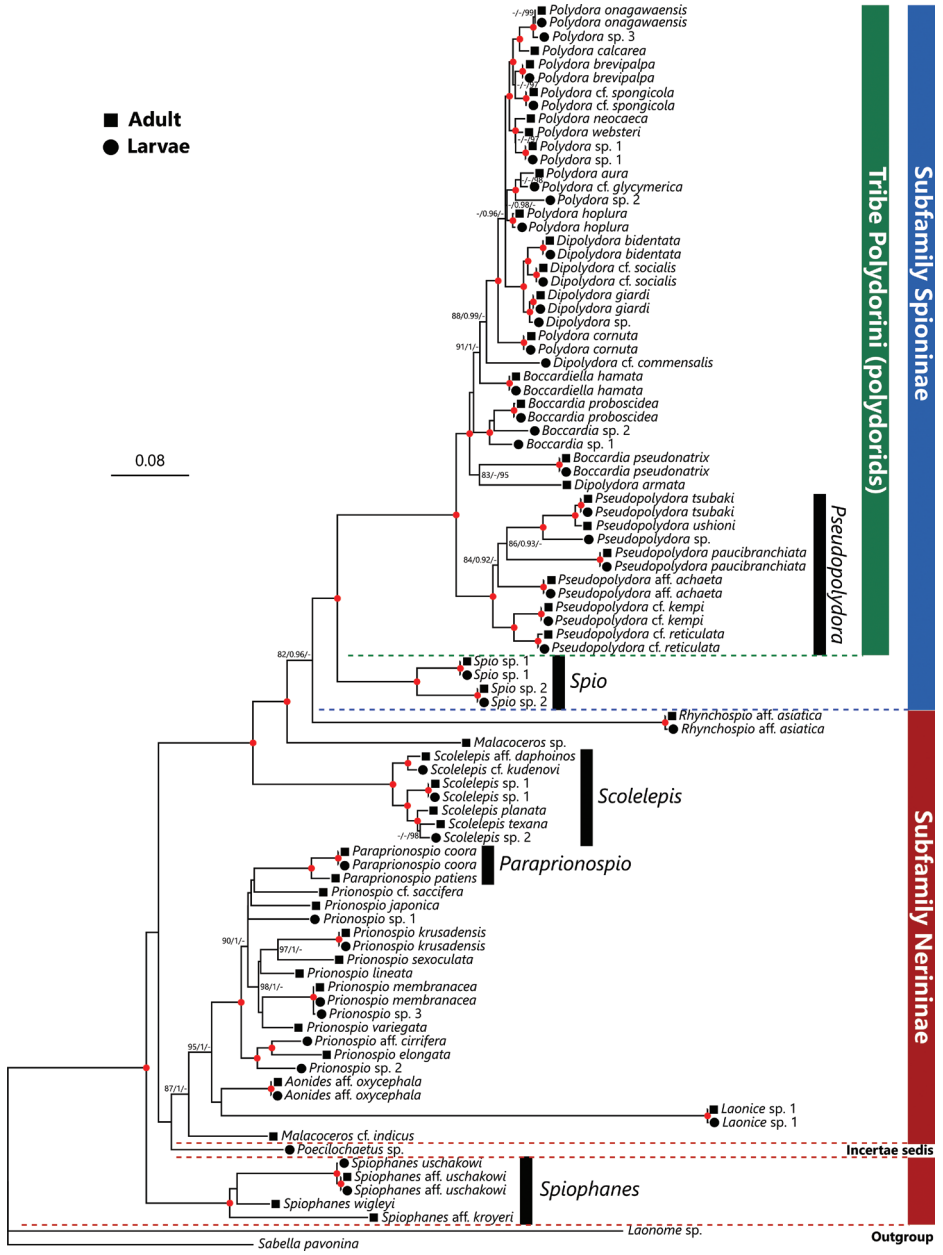


**Figure 2.** Maximum Likelihood tree inferred from nuclear 18S and mitochondrial 16S rRNA gene sequences of spionids obtained from Japan in the present and previous studies (provided in Table 1). The gene sequences of adult and larval spionid polychaetes are indicated by solid squares and circles in front of each species name, respectively. SH-aLRT/approximate Bayes support/ultrafast bootstrap support values of ≥ 80%, ≥ 0.95, ≥ 95%, respectively are given beside the respective nodes. Nodes with red circles indicate triple high support values of SH-aLRT ≥ 80, approximate Bayes support ≥ 0.95, and ultrafast bootstrap support ≥ 95. The scale bar represents the number of substitutions per site. Sequences of *Laonome* sp. and *Sabella pavonina* Savigny, 1822 obtained from DDBJ/EMBL/GenBank were used for outgroup rooting.

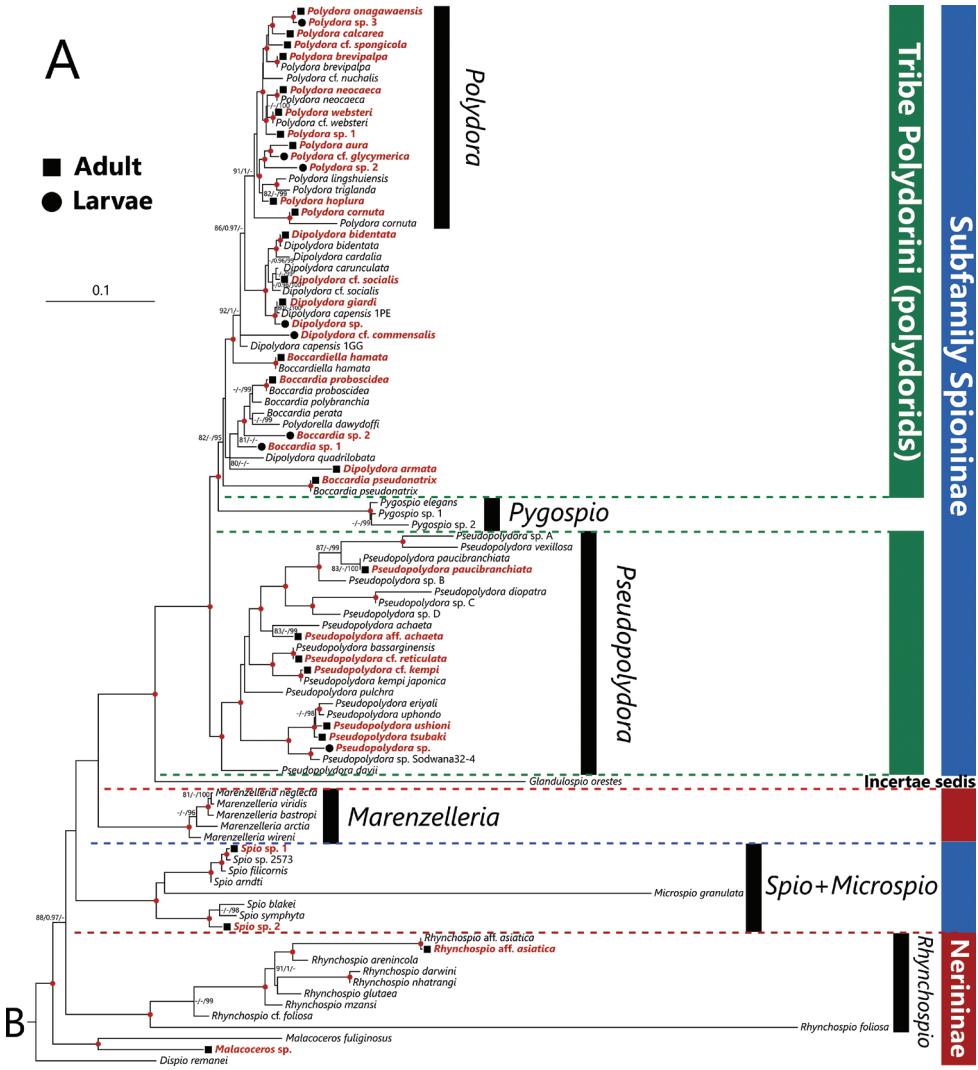


**Figure 3.** Maximum Likelihood tree inferred from nuclear 18S and mitochondrial 16S rRNA gene sequences of spionid polychaetes obtained from Japan in the present and previous studies (shown in Table 1) and from DDBJ/EMBL/GenBank (shown in Table 2). The tree is divided into two parts **A, B**. The gene sequences obtained in the present study are highlighted by bold and red color and the adult and larval sequences are indicated by solid squares and circles in front of each species name, respectively. SH-aLRT/approximate Bayes support/ultrafast bootstrap support values of ≥80%/≥0.95/≥95%, respectively are given beside the respective nodes. Nodes with red circles indicate triple high support values of SH-aLRT ≥ 80, approximate Bayes support ≥ 0.95, and ultrafast bootstrap support ≥ 95. The scale bar represents the number of substitutions per site. Sequences of *Laonome* sp. and *Sabella pavonina* Savigny, 1822 obtained from GenBank were used for outgroup rooting.

ogy of late spionid larvae from northeastern Japan are provided at the beginning of the larval morphological description section of each genus. An identification key to genera is provided below.

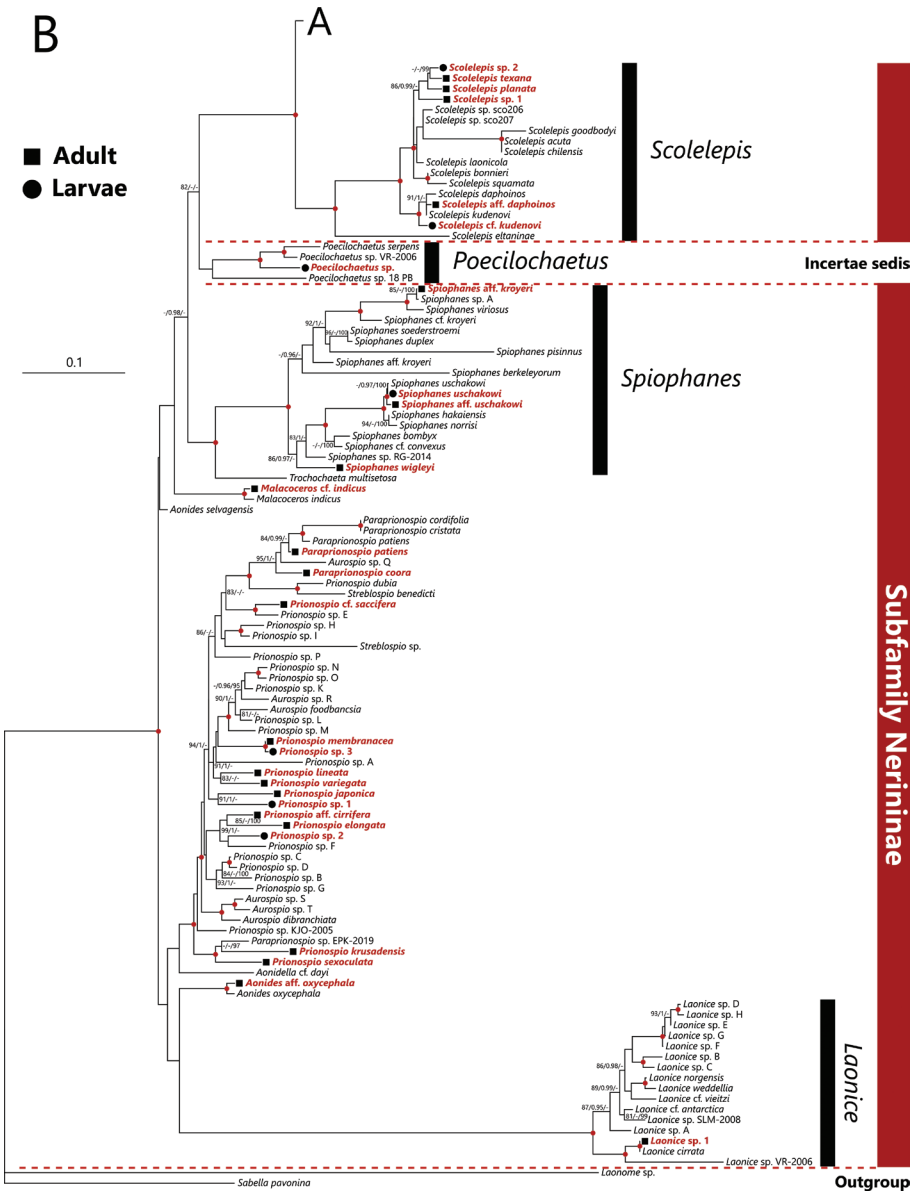


**Figure 3.** Continued.

## Identification key to genera of the spionid larvae in northeastern Japan

1 Two pairs of red or dark red eyes present; distinct black pigmentation or melanophore (branching black chromatophores) absent; gastrotrochs from chaetiger II or III onwards in all following chaetigers (except *Rhynchospio*: not in all following chaetigers) ..... **[subfamily Nerininae] 2**

– Three pairs of black eyes present (most lateral two pairs of eyes often double-eyes: see Hacker 1896; Hannerz 1956); distinct black pigmentation present;

| | |
|---|---|
| | gastrotrochs from chaetiger III, V, or VII onwards not in all following chaetigers ..... **[subfamily Spioninae] 8** |
| – | Two pairs of dark red eyes present; small black pigmentation present laterally between parapodia on every chaetigers; gastrotrochs from chaetiger I onwards in all following chaetigers..... **Genus *Poecilochaetus*** |
| 2 | Lateral parts of peristomium well developed and distinctly demarcated from prostomium ..... **3** |
| – | Lateral parts of peristomium less developed and less demarcated from prostomium..... **5** |
| 3 | Prostomium not pointed anteriorly, more or less stumpy; lateral parts of peristomium not clearly demarcated ..... **4** |
| – | Prostomium pointed anteriorly and tip of prostomium terminates in a tapered tip; lateral parts of peristomium clearly demarcated as large peristomial umbrella ..... **Genus *Scolelepis*** |
| 4 | Parapodia well differentiated; long larval chaetae only in notopodia..... ..... **Genus *Rhynchospio*** |
| – | Parapodia less differentiated; serrated larval chaetae occur in both noto- and neuropodia ..... **Genus *Laonice*** |
| 5 | Slender, moderately long in overall shape; body not transparent; some pigmentation of various colors on pharynx, proctodaeum, prostomium, peristomium, pygidium, and/or various locations of the body in late larvae ..... **6** |
| – | Slender, fairly long in overall shape with numerous chaetigers; body nearly transparent; pigmentation almost completely absent except red or green pigmentation on pharynx and/or pygidium ..... 7 |
| 6 | Body not rich in yolk; larval chaetae on first chaetiger medium length; pharynx not colored in black; prostomium rounded anteriorly ..... **Genus *Spiophanes*** |
| – | Body rich in yolk; larval chaetae on first chaetiger fairly long especially in early larvae; pharynx colored in black; prostomium rectangular anteriorly (prostomium extended and tapered anteriorly in juvenile) .... **Genus *Aonides*** |
| 7 | Prostomium anteriorly rounded; lateral parts of peristomium relatively demarcated from prostomium; quite large and long larvae with well-developed branchiae in late larvae..... **Genus *Paraprionospio*** |
| – | Prostomium anteriorly rounded; lateral parts of peristomium less demarcated from prostomium; long and slender larvae with no or less developed branchiae..... **Genus *Prionospio*** |
| 8 | Overall body shape long and slender..... **9** |
| – | Overall body shape thick/slender and fusiform ..... **11** |
| 9 | Modified chaetae in chaetiger V present in late larvae; larval chaetae on first chaetiger medium length ..... **10** |
| – | Modified chaetae in chaetiger V absent; larval chaetae on first chaetiger fairly long ..... **Genus *Spio*** |
| 10 | Pairs of large branching dorsal melanophores present..... **Genus *Polydora*** |
| – | Pairs of large branching dorsal melanophores absent..... **Genus *Dipolydora*** |

11 Lateral prostomium expansion moderate (except for *Boccardia* sp. 2); mid-dorsal melanophores arranged in a single row (except *B. pseudonatrix*); vestibule or pharynx pigmented with black or brown..... **Genus *Boccardia***

– Lateral prostomium expand greatly; arrangement of dorsal melanophores not arranged in a single row; vestibule or pharynx not pigmented with black or brown .....**12**

12 A mid-dorsal branching melanophore on first chaetiger absent; more than two pairs of dorsal black pigmentation spots/bands on each chaetiger..... ..... **Genus *Boccardiella***

– A mid-dorsal branching melanophore on first chaetiger present (except for *Pseudopolydora* cf. *kempi*: mid-dorsal melanophore on first chaetiger usually absent); one or two pairs of dorsal melanophores on each chaetiger ..... .....**Genus *Pseudopolydora***

## Description of larval morphology

**Family SPIONIDAE Grube, 1850**
**Subfamily NERININAE Söderström, 1920**

### Genus *Aonides* Claparède, 1864

**Larval diagnosis.** The overall shape slender. Prostomium rounded or rectangular anteriorly. The lateral parts of the peristomium more or less demarcated from prostomium. Two pairs of red eyes present. Melanophore absent, some brown or dark pigmentation may be present in pharynx and pygidium. Larval chaetae coarsely or slightly serrated. Larval chaetae in first chaetiger very long, extend beyond pygidium in late trochophore and early nectochaete stages. Nototrochs develop in late larval stages. Gastrotrochs occur in all chaetigers from chaetiger II onwards. Two pairs of pygidial cirri develop in late larval stage. The body of early larvae covered by egg envelope, yellowish opaque appearance with abundant yolk. Two parallel rows of encircling vesicles of egg envelope present in pretrochophore and trochophore stages. Holopelagic lecithotrophic development unique among spionids (Hannerz 1956; Blake and Arnofsky 1999, as *Dispio uncinata* Hartman, 1951: see Radashevsky et al. 2011; Blake 2006, as *D. uncinata*).

### *Aonides* aff. *oxycephala* (Sars, 1862)

Fig. 4A–C

**Larval morphology.** Remnants of egg envelope apparent in early trochophore (Fig. 4A). In ten-chaetiger larvae, egg envelope becomes incorporated into larval cuticle, two pairs of red eyes arranged in an approximately straight line (Fig. 4B). Larval chaetae on first chaetiger long especially in early larvae (Fig. 4A, B). Late larvae long and slender in shape (Fig. 4C). Prostomium rectangular anteriorly in larval stages, considerably ex-

tended and tapered in juvenile stage. Lateral parts of peristomium moderately demarcated from prostomium. Black pigment in pharynx. Pigmentation absent except in the eyes and pharynx. Pygidium acquires two pairs of dorsal cirri in late larvae (Fig. 4C).

**Remarks.** Adult individuals of this species were collected from muddy bottom sediment at 22 m depth in Onagawa Bay in January 2011 and 2012 using a Smith-McIntyre grab sampler. Adult morphology agrees with the description of *A. oxycephala* by Imajima (1989). *Aonides oxycephala* originally described from Norway has been reported worldwide and is considered cosmopolitan. However, these reports may comprise a series of similar or sibling species, as pointed out by Radashevsky (2015). The gene sequences obtained in the present study were 100% match in 18S rRNA but 8.6% (29/337 bp) different in 16S rRNA from that of *A. oxycephala* from France (MG913226 and MG878895). Therefore, the species collected in the present study was referred to *A.* aff. *oxycephala*. The larvae and adults were confirmed to match (18S: 1753/1753, 16S: 447/448 bp) using molecular data (Fig. 2).

Planktonic larvae were found in Onagawa Bay from October to December. In early larval stages, the larvae of this species are similar to those of *Laonice* sp. (Fig. 4D); but larval chaetae are longer, and the body is yolkier and opaquer in this species. The larval morphology of *A.* aff. *oxycephala* is similar to that of *A. oxycephala* described by Hannerz (1956). However, the peristomium of the former species is more developed and demarcated from the prostomium compared to the latter. Black pigmentation of the pharynx in late larval stages was not reported by Hannerz (1956).

### Genus *Laonice* Malmgren, 1867

**Larval diagnosis.** Overall shape short, thick, and fusiform. Prostomium stumpy, rectangular, notched anteriorly. Lateral parts of peristomium clearly demarcated from prostomium. The short palps attached to outer end of lateral parts of peristomium. Two pairs of red eyes present. Melanophores and pigmentation absent except eyes. Nototrochs absent. Gastrotrochs occur in all chaetigers from chaetiger III onwards. Well-developed serrated larval chaetae occur both in noto- and neuropodia, notochaetae characteristically introverted toward medial line of dorsal side. Early larvae covered by egg envelope (Hannerz 1956; Plate and Husemann 1994).

#### *Laonice* sp. 1

Fig. 4D

**Larval morphology.** Remnants of egg envelope apparent in early trochophore (Fig. 4D). Two pairs of red eyes located in approximately a straight line, lateralmost pair larger in early larvae. Parapodia weakly differentiated; serrated larval chaetae introverted toward medial line of dorsal side. The body opaque yellowish with abundant yolk internally. Pigmentation absent except in the eyes.

**Remarks.** Adult individuals of this species were collected from bottom sediments at 22 m depth in Onagawa Bay in December 2011 using a Smith-McIntyre grab sampler. To date, two *Laonice* species, *L. cirrata* (Sars, 1851) and *L. japonica* (Moore, 1907) have been recorded from Japan. Sikorski (2002) indicated that *L. cirrata*, a previously presumed widespread species, is probably limited to Norway and adjacent regions. This was supported by a molecular study that suggested previously unrecognized diversity within this species (Bogantes et al. 2018). *Laonice japonica*, originally described as *Spionides japonicus* from Japan and later considered as synonymous with *L. cirrata* (e.g., Söderström 1920; Berkeley and Berkeley 1936; Okuda 1937; Imajima and Hartman 1964; Foster 1971), was reexamined and considered a valid species by Maciolek (2000) and Sikorski (2011). However, even after that, since *L. cirrata* has been recorded from Japan (e.g., Imajima 2006, 2009, 2011), the validity of these records is ambiguous and might represent different species. In addition to these two species, unidentified *Laonice* sp. was also reported from Japan by Imajima (1990c) but it is unclear whether the species is identical to the species reported here. Although the 18S rRNA gene sequences obtained in the present study match (1731/1731 bp, except for gaps) with *Laonice cirrata* sequences from Russia in DDBJ/EMBL/GenBank (KM998754), because taxonomic knowledge on this genus in Japan is still limited and the 18S rRNA gene is relatively conservative, this species was referred to *Laonice* sp. The larvae and adults were confirmed to match (18S: 1754/1754, 16S: 500/504 bp) using molecular data (Fig. 3).

Larvae of this species were rare in the planktonic community found in Onagawa Bay in September 2011 and October 2012. Although two parallel rows of encircling vesicles of egg envelope, similar to those of *Aonides* pretrochophore and trochophore stages, were reported in oocytes of *Laonice* species (Radashevsky and Lana 2009), this characteristic was not observed in early larval stages with egg envelope in the present study (Fig. 4D). Blake (2006) described the larval development of *Laonice* sp. from California; however, the identification of these larvae is doubtful because they seem to lack serrated larval notochaetae introverted toward the medial line of the dorsal side, which are characteristic of *Laonice* larvae.

### *Laonice* sp. 2

Fig. 4E

**Larval morphology.** Late larvae thick and stumpy in shape (Fig. 4E). Prostomium stumpy, somewhat notched at tip. Lateral parts of peristomium well demarcated from prostomium. In late larvae, two pairs of red eyes are arranged in a trapezoidal shape, the medial pair bigger and situated anteriorly. Short palps developed in late larvae, attached to outer end of lateral parts of peristomium. Parapodia weakly differentiated; serrated larval chaetae in both noto- and neuropodia; notochaetae characteristically introverted toward medial line of dorsal side. Gut dark green in color internally. Pigmentation absent except in the eyes.


**Figure 4.** Light micrographs showing morphologies of living spionid larvae of *Aonides*, *Laonice*, *Rhynchospio*, *Scolelepis*, and *Spiophanes* **A–C** *Aonides* cf. *oxycephala*, dorsal view of early planktonic (**A**), 8-chaetiger (**B**), and 18-chaetiger larvae (**C**) **D** *Laonice* sp. 1, dorsal view of early planktonic larva **E** *Laonice* sp. 2, dorsal view of 12-chaetiger larva **F** *Poecilochaetus* sp., dorsal view of 17-chaetiger larva **G, H** *Rhynchospio* aff. *asiatica*, dorsal view of 6-chaetiger (**G**) and 12-chaetiger larvae (**H**) **I** *Scolelepis* cf. *kudenovi*, dorsal view of 7-chaetiger larva **J, K** *Scolelepis* sp. 1, dorsal view of 17-chaetiger (**J**) and 19-chaetiger larvae (**K**) **L** *Scolelepis* sp. 2, dorsal view of 20-chaetiger larva **M, N** *Spiophanes uschakowi*, dorsal (**M**) and lateral view (**N**) of 18-chaetiger larvae **O, P** *Spiophanes* aff. *uschakowi*, dorsal view of 16-chaetiger larva (**O**) and 27-chaetiger juvenile (**P**). Scale bars: 300 μm.

**Remarks.** Adult individuals of this species were not collected in the present study. Only one individual of larva of this species were collected in Habu Port in June 2016. Even though the 18S and 16S rRNA gene sequences were not obtained, the larvae were identified as belonging to *Laonice* because the larval morphology of this species agrees with that of *L. cirrata* described by Hannerz (1956) and *L.* cf. *cirrata* described by Plate and Husemann (1994). However, the hooded hooks in neuropodia described by Hannerz (1956) were not observed in the specimens of the present study (nor in those reported by Plate and Husemann [1994]); this may be because the larvae collected here were less developed and the hooks were reported only from chaetiger XIV onwards.

### Genus *Paraprionospio* Caullery, 1914

**Larval diagnosis.** Overall shape long and slender, large in size (> 4 mm) and number of chaetigers (> 35 chaetigers) at metamorphosis. Prostomium rounded. Lateral parts

of peristomium moderately demarcated from prostomium. Two pairs of red or dark red eyes present. Pigmentation absent except eyes and some reddish pigmentation on pygidium. Nototrochs absent. Gastrotrochs occur in all chaetigers from chaetiger II onwards. Branchiae well developed and elongated in late larvae. Long larval chaetae may be absent in chaetiger II (Berkeley and Berkeley 1961, as *Prionospio*; Carrasco 1976; Yokoyama 1981, 1996; Blake and Arnofsky 1999).

### *Paraprionospio coora* Wilson, 1990

Fig. 5A, B

**Larval morphology.** Long and thin in shape, quite large and long body with numerous chaetigers. Prostomium anteriorly rounded, lateral lips elevated from the ventrolateral side of prostomium (Fig. 5A). Late larvae acquire caruncle extending posteriorly from posterior part of prostomium (Fig. 5B). Peristomium fuses with the first larval segment at late larval stage (Fig. 5B). First pair of branchiae well developed, branchial pinnation still absent. Two pairs of red eyes arranged in somewhat trapezoidal shape, lateral pair kidney-shaped, situated anteriorly. Posterior part of pygidium pigmented reddish brown, anal cirri develop in late larvae.

**Remarks.** Adult individuals of this species were collected from muddy bottom sediments at 22 m depth in Onagawa Bay in December 2011 by using a Smith-McIntyre grab sampler. Adult morphology agrees with the description of *P. coora* by Yokoyama (2007), and therefore this species was referred to *P. coora*. The larvae and adults were confirmed to match (18S: 1754/1754, 16S: 500/500 bp) using molecular data (Fig. 2).

Only three planktonic larvae of this species were found in Onagawa Bay in November 2011 and Sasuhama in January 2013. The morphological characteristics and size of these larvae are similar to those in previous descriptions of the species from the same genus (Yokoyama 1981, 1996). However, the larvae of *P. coora* lack red pigmentation on the dorsolateral side of the lateral lips, which characterizes the larvae of *Paraprionospio patiens* Yokoyama, 2007 (Yokoyama 1981; as *P. pinnata*: see Yokoyama 2007). Additionally, lamellae of the first pair of branchiae in *P. coora* are less developed in late larvae with more than 30 chaetigers (Fig. 5B) compared with the larvae of *P. patiens* (Yokoyama 1981) and *Paraprionospio cordifolia* Yokoyama, 2007 (Yokoyama 1996, as *Paraprionospio* sp. form B: see Yokoyama 2007). Yokoyama (1981) suggested that the larvae of *Paraprionospio* are the largest in size and number of chaetigers at metamorphosis among the spionid larvae. However, late larvae of *Poecilochaetus* exceeding 5 mm (Magalhães et al. 2015) and with more than 40 chaetigers are often reported (Hannerz 1956; Plate and Husemann 1994).

## Genus *Poecilochaetus* Claparède in Ehlers, 1875

**Larval diagnosis.** Overall shape long and slender, large in size (> 5 mm) and number of chaetigers (> 30 chaetigers) at metamorphosis. Body transparent, characterized by

total absence of pigmentation except pairs of small pigment spot between parapodia or ventro-lateral side of each chaetiger. Two pairs of red or dark red eyes present. Gastrotrochs from chaetiger I onwards in all following chaetigers, gastrotrochs in first and second chaetigers represented by solitary lateral patches of cilia and complete gastrotrochs occur from third chaetiger onwards. Nototrochs absent. Larvae prior to ca. 30–40 chaetiger stages remain in metatrochophore stage, characterized by absence of functional parapodia for swimming and presence of well-developed proto-, telo-, and gastrotrochs for swimming. Metatrochophore have broadened trapezoidal prostomium with tactile cilia in anterior part, broad and low caruncle, provisional larval chaetae, pygidium without anal cirri. Larval stage after metatrochophore stage (often called nectosoma) characterized by reduced trochs, the presence of functional parapodia, and rapid serpentine swimming behavior. In nectosoma stage, caruncle, nuchal lobes, a pair of palps, parapodia, cirriform or digitiform dorsal and ventral postchaetal lobes, and two pairs of anal cirri on pygidium develop gradually (Thorson 1946; Hannerz 1956; Berkeley and Berkeley 1961; Reddy and Mohan 1982; Plate and Husemann 1994; Magalhães et al. 2015).

### *Poecilochaetus* sp.

Fig.4F

**Larval morphology.** Overall shape long and slender. Two pairs of dark red eyes present. Metatrochophore larvae with 17 chaetigers have broadened trapezoidal prostomium with tactile cilia in anterior part, broad and low caruncle, provisional larval chaetae, and well developed prototrochs (Fig. 4F). Body of metatrochophore larvae transparent, characterized by small pigment spot between parapodia from chaetiger II onwards. Pygidium without anal cirri. Incomplete anterior fragment of nectosoma larvae characterized by extremely long body, rounded prostomium, broad and low caruncle, reduced prototrochs, parapodia with digitiform dorsal postchaetal lobes, and rapid serpentine swimming behavior. The body of nectosoma larvae transparent and small pigment spot laterally on each side of chaetigers. Occipital antenna and pair of palps not observed.

**Remarks.** Adult individuals of this species were not collected in the present study. Even though the 18S and 16S rRNA gene sequences obtained from larvae in the present study did not match any of the *Poecilochaetus* sequences from DDBJ/EMBL/GenBank, this species was referred to *Poecilochaetus* sp. because specimens formed a monophyletic clade with the other *Poecilochaetus* species with robust statistical support (Fig. 3).

Planktonic larvae of this species were collected in Onagawa Bay in August 2010 and January 2013. The larval morphology of this species is similar to that of *Poecilochaetus serpens* Allen, 1904 described by Hannerz (1956) and Plate and Husemann (1994). However, the former species differs from the latter by not having yellow chromatophores on the "head" and pygidium as described by Plate and Husemann (1994). The

pigmentation pattern of *Poecilochaetus* sp. larvae also differs from that of *Poecilochaetus anterospinus*, which has a pair of ventral green melanophores on the lateral side of each segment, beginning from chaetiger IV throughout (Magalhães et al. 2015).

### Genus *Prionospio* Malmgren, 1867

**Larval diagnosis.** Overall shape long and slender. Prostomium rounded anteriorly. Lateral parts of peristomium not demarcated from the prostomium. Two pairs of red or dark red eyes present. Pigmentation usually absent except for eyes and on pygidium. Some species (e.g., *P. steenstrupi* and *P. krusadensis*) have red or green pigmentation on pharynx, dorsal side, and/or pygidium. Nototrochs absent or occur in branchial chaetigers in late larvae. Gastrotrochs occur in all chaetigers from chaetiger II–IV onwards (Thorson 1946, as *Disoma*; Hannerz 1956; Plate and Husemann 1994; Radashevsky et al. 2006).

#### Identification key to species of the larvae belonging to the genus *Prionospio* in northeastern Japan

1 Green pigmentation on pharynx and pygidium present ..... ***Prionospio krusadensis***

– Green pigmentation on pharynx and pygidium absent ..... ***Prionospio membranacea* or *Prionospio* spp. 1 and 2**

#### *Prionospio krusadensis* Fauvel, 1929

Fig. 5C, D

**Larval morphology.** Long and thin in shape. Prostomium rounded anteriorly, lateral parts of the peristomium not especially well demarcated. Two pairs of red eyes arranged somewhat in trapezoidal shape, lateral pair kidney-shaped and situated anteriorly. Body extremely transparent (Fig. 5D). Green pigmentation on pharynx and pygidium (Fig. 5C, D). Caruncle develop in late larvae, extends posteriorly from posterior part of prostomium (Fig. 5C). In late larvae, branchial anlages occur from chaetiger II, pygidium acquires anal cirri.

**Remarks.** Adult individuals of this species were collected from shallow subtidal muddy bottom sediments in Sasuhama in August 2011 by using a hand-scoop. The species was referred to *P. krusadensis* as the adult morphology agrees with the descriptions of this species by Imajima (1990a). The larvae and adults were confirmed to match (18S: 1749/1749, 16S: 506/507 bp) using molecular data (Fig. 2).

Planktonic larvae of this species were found in Sasuhama and Onagawa Bay in July and August, but they were rare in the plankton samples. Green pigmentation on the pharynx and pygidium is characteristic of the larvae of this species.

***Prionospio membranacea* Imajima, 1990**
Fig. 5E–G

**Larval morphology.** Long and thin in shape. Prostomium rounded anteriorly, lateral parts of peristomium not especially well demarcated. Two pairs of red or dark red eyes arranged somewhat in trapezoidal shape, lateral pair kidney-shaped and situated anteriorly. Small caruncle develop in late larvae, extends posteriorly from posterior part of prostomium (Fig. 5G). In late larvae branchial anlages occur from chaetiger II, pygidium acquires anal cirri (Fig. 5G). Palps not yet developed in 15- and 20-chaetiger larva (Fig. 5E, F) but developed in 24-chaetiger larva (Fig. 5G). Pigmentation absent except eyes.

**Remarks.** Adult individuals of this species were collected from muddy bottom sediments at 22 m depth in Onagawa Bay in December 2011 by using a Smith-McIntyre grab sampler. The species was referred to *P. membranacea* as the adult morphology agrees with the descriptions of this species by Imajima (1990b). The larvae and adults were confirmed to match (18S: 1752/1752 and 1747/1750 except for gaps, 16S: 502/505 bp) using molecular data (Fig. 2). Planktonic larvae of this species were found in Onagawa Bay during August to October.

***Prionospio* spp. 1 and 2**
Fig. 5H, I

**Larval morphology.** Long and thin in shape. Prostomium rounded anteriorly, lateral parts of the peristomium not especially well demarcated. Two pairs of red or dark red eyes arranged somewhat in trapezoidal shape, lateral pair kidney-shaped and situated anteriorly. Small caruncle develop in late larvae, extends posteriorly from posterior part of the prostomium. In *Prionospio* sp. 2, branchial anlages occur from chaetiger II, pygidium acquires anal cirri (Fig. 5I). Pigmentation absent except eyes in *Prionospio* sp. 1. Gut pigmented in orange in *Prionospio* sp. 2. Palps developed in 19-chaetiger larva of *Prionospio* sp. 2.

**Remarks.** Two unidentified species of planktonic larvae of the genus *Prionospio* other than *P. krusadensis* and *P. membranacea* were collected from Onagawa Bay. The adult individuals of these species were not collected in the present study. Even though the 18S and 16S rRNA gene sequences obtained from these larvae in the present study did not match any *Prionospio* sequences obtained in the present study nor with those registered in DDBJ/EMBL/GenBank, these species were referred to *Prionospio* sp. 1 and 2 as the larvae were similar to the other *Prionospio* species in their morphology and gene sequences (Figs 2, 3). The larvae of *Prionospio* sp. 1 and 2 are similar to each other and to that of *P. membranacea*, and it is difficult to distinguish among them based only on their morphology.

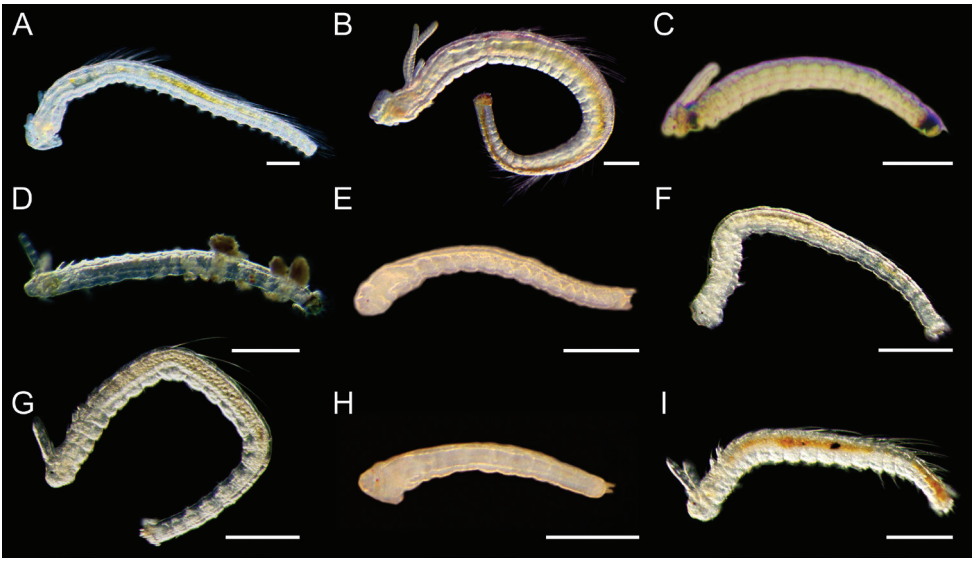


**Figure 5.** Light micrographs showing the morphologies of living spionid larvae of genera *Paraprionospio* and *Prionospio* **A, B** *Paraprionospio coora*, lateral view of 25-chaetiger (**A**) and 33-chaetiger larvae (**B**) **C, D** *Prionospio krusadensis*, lateral view of 17-chaetiger larvae **E–G** *Prionospio membranacea*, lateral view of 15-chaetiger (**E**), 20-chaetiger (**F**), and 24-chaetiger larvae (**G**) **H**, *Prionospio* sp. 1, lateral view of 11-chaetiger larva **I** *Prionospio* sp. 2, lateral view of 19-chaetiger larva. Scale bars: 300 μm.

### Genus *Rhynchospio* Hartman, 1936

**Larval diagnosis.** Overall body shape short and thick. Prostomium broad and straight or slightly notched anteriorly. Lateral parts of peristomium clearly demarcated from prostomium. Palps attached to outer end of lateral parts of peristomium. Two pairs of red or black eyes present. Faint yellow pigment may be present in anterior part of prostomium and posterior part of pygidium. In late larvae, pair of prominent antero-lateral processes on prostomium developed. Melanophore absent, black or yellowish pigmentation occur in some species. Nototrochs weakly developed, occur in all chaetigers except first chaetiger. Gastrotrochs occur regularly in every other chaetiger from chaetiger III onwards (Carrasco 1976; Radashevsky 2007).

### *Rhynchospio* aff. *asiatica* sensu Radashevsky et al., 2014

Figs 4G, H, 6

**Larval morphology.** Overall body shape short and thick in relation to length. Prostomium broad, stumpy, somewhat notched anteriorly. Peristomium well developed, forming wide collar on sides of prostomium. Two pairs of red or dark red eyes arranged

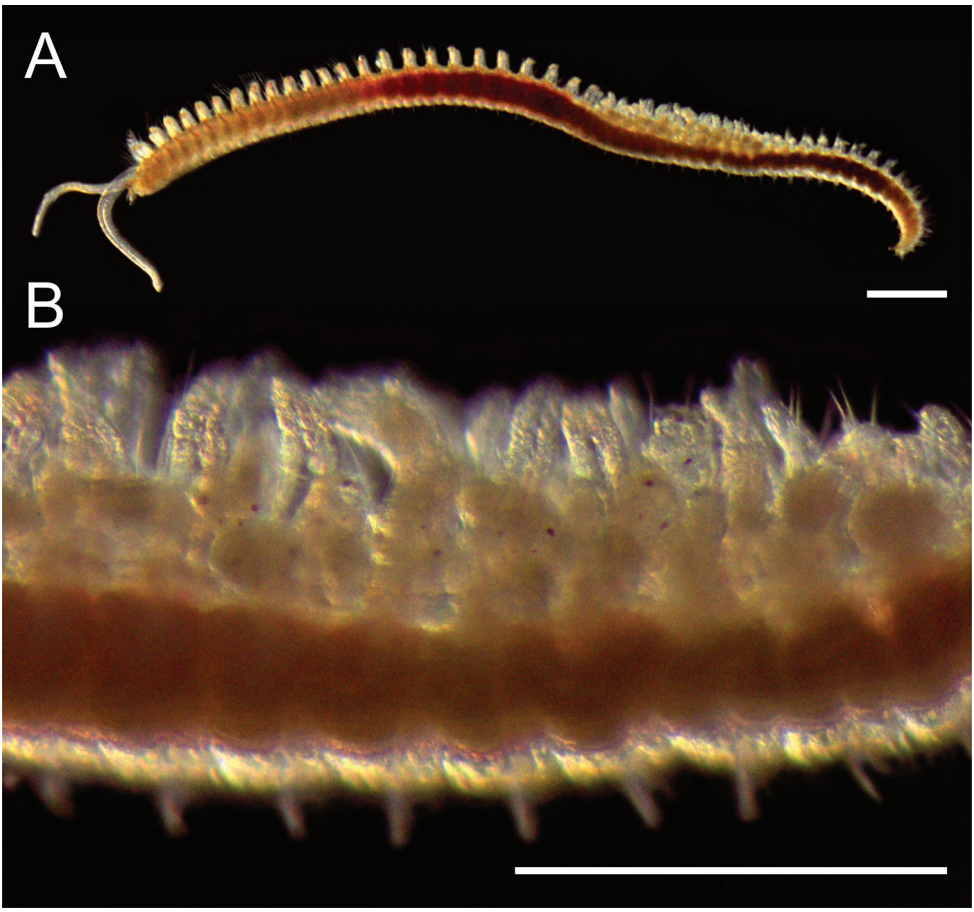


**Figure 6.** Light micrographs showing dorsal brooding of *Rhynchospio* aff. *asiatica*. Hermaphroditic individual broods their larvae between dorsal branchiae on posterior chaetigers. Scale bars: 1 mm.

in straight line, lateral pair in kidney-shape. Parapodia strongly differentiated in late larvae. Larval chaetae occur only in notopodia. Pygidium large and round, acquires dorsal cirri in late larvae. Late larvae have two antero-lateral processes on prostomium (Fig. 4H). Pigmentation usually absent, some individuals have brownish pigmentation on peristomium, dorsum, and/or pygidium, and/or two medial black pigmentation ventrally on approximately chaetiger VI and anterior margin of the pygidium.

**Remarks.** Adult individuals of this species were collected from intertidal and shallow subtidal sandy or muddy bottom sediments in Gamo Lagoon in January 2011 and Sasuhama in September 2011 by using a hand-scoop. To date, three *Rhynchospio* species, *R. foliosa* Imajima, 1991, *R. gutaea* (Ehlers, 1987), and *R. tuberculata* Imajima, 1991, have been recorded from Japan (Imajima 1991a). However, extensive morphological and molecular studies revealed the absence of records of *R. glutaea* from the

northern Pacific Ocean (Radashevsky 2007; Radashevsky et al. 2014, 2016a). Radashevsky et al. (2014) also referred to *R. arenicola* Hartman, 1936, *R. asiatica* Chlebovisch, 1959, *R.* aff. *asiatica*, and *R. glutaea* as members of the *R. glutaea* complex because they resembled each other so closely. Adult morphology and 18S and 16S rRNA gene sequences of *Rhynchospio* specimens obtained in the present study agree (18S: 1716/1716, 16S: 486/492 bp) with those of *R.* aff. *asiatica* (Fig. 3) from South Korea (KJ546296) reported by Radashevsky et al. (2014); therefore, this species was referred to *R.* aff. *asiatica* sensu Radashevsky et al. (2014). The larvae and adults were confirmed to match (18S: 1783/1783, 16S: 471/477 bp) using molecular data (Fig. 2).

This species is recorded from Japan for the first time in the present study. The brooding of larvae beneath dorsal branchiae in this species was observed in September 2011 (Fig. 6). The larvae adhere to their parents and are enclosed by branchiae present on the posterior chaetigers (26$^{th}$–39$^{th}$ chaetigers). The larvae are retained on the parents' dorsum even when the parent individuals leave their tube, unless the parent is disturbed. Larvae seemed to be released at around the 3-chaetiger stage; the fact that planktonic larvae with more than three chaetigers were commonly collected from plankton supports this observation. Similar dorsal larval brooding was reported in other *Rhynchospio* species (Levin 1982; Radashevsky 2007; Radashevsky et al. 2014) and in *Streblospio benedicti* Webster, 1879 (Levin 1982, 1984).

Planktonic larvae were found in Onagawa Bay, Gamo Lagoon, and Sasuhama in almost every season of the study period, but few were found in winter season (November to March). Larval morphology of this species resembles that of *R. glutaea* and *R. nhatrangi* Radashevsky, 2007 described by Carrasco (1976) and Radashevsky (2007), respectively. The overall larval morphology of *Rhynchospio* species is quite similar to that of the genus *Malacoceros* described in Hannerz (1956, as *Scolelepis*), but it differs in the latter having three pairs of black eyes, the most lateral pairs with double-eyes.

### Genus *Scolelepis* Blainville, 1828

**Larval diagnosis.** Overall shape thick and fusiform. Prostomium pointed anteriorly, terminates in retractile, muscular tip. Lateral parts of peristomium clearly demarcated from prostomium, forming large peristomial umbrella. Short palps on lateral-most parts of peristomium. Two pairs of red eyes present. Melanophore absent, black, brown, orange, red, or green pigmentation patches often present in body surface, pharynx, gut, and/or proctodaeum. Nototrochs present or absent. Gastrotrochs occur in all chaetigers from chaetiger II or III onwards. Pygidium large, inflated, and surrounded by thick telotroch (Okuda 1946, as *Spio filicornis*; Hartman 1941, as *Nerinides*; Thorson 1946, as *Nerine* in part see Hannerz 1956; Hannerz 1956, as *Nerine* and *Nerinides*; Imajima 1959, as *Nerinides*; Dean and Hatfield 1963, as *Nerinides*; Carrasco 1976, as *Nerine* and *Nerinides*; Plate and Husemann 1994; Scheltema et al. 1997; Blake and Arnofsky 1999; Blake 2006).

## Identification key to species of the larvae belonging to the genus *Scolelepis* in northeastern Japan

1 Pharynx and pygidium colored green; pygidium very broad and horseshoe-shaped ..... ***Scolelepis* cf. *kudenovi***
– Pharynx pigmented orange and gut pigmented brown; Pygidium broad and spherical shaped ..... **2**
2 Prostomium sharply tapered anteriorly; gut diverticula not strongly segmented ..... ***Scolelepis* sp. 1**
– Prostomium bluntly tapered anteriorly; gut diverticula strongly segmented.... ..... ***Scolelepis* sp. 2**

### *Scolelepis* cf. *kudenovi* Hartmann-Schröder, 1981

Fig. 4I

**Larval morphology.** Thick and fusiform in shape. Prostomium pointed anteriorly, terminates in retractile, muscular tip. Lateral parts of peristomium clearly demarcated from prostomium, forming peristomial umbrella. Peristomial umbrella carrying well-developed prototroch. Two pairs of red eyes arranged in somewhat trapezoidal shape, medial pair situated anteriorly. Greenish pigment in pharynx and proctodaeum. Pygidium very broad and horseshoe-shaped.

**Remarks.** Only three individuals of early larvae of this species were collected from Sasuhama in January 2012. The 18S rRNA gene sequences obtained in the present study for these specimens match (464/464 bp) that of *S. kudenovi* from Lizard Island, Australia (KP636517: Meißner and Götting 2015). Since the species identification is unreliable because of the short reference sequence, this species was referred to *S.* cf. *kudenovi*.

The sequence of an adult individual, which collected from the surf zone of the sandy beach in Rishiri Island and previously identifies as *Scolelepis kudenovi* (Abe et al. 2019c) as the morphology agrees with the descriptions of *S. kudenovi* by Imajima (1992) and Meißner and Götting (2015), 100% matched with that of the larvae of *Scolelepis* cf. *kudenovi* in 18S rRNA gene (1819/1819 bp) but largely differed in 16S rRNA gene (462/505 bp). Because the 16S rRNA gene of the adult individual was rather closer to *S. daphoinos* (430/455 bp) from China (GU362676, Zhou et al. 2010), it is referred to *S.* aff. *daphoinos* in the present study (Table 1, Figs 2, 3).

### *Scolelepis* sp. 1

Fig. 4J, K

**Larval morphology.** Thick and fusiform in overall shape. Prostomium pointed anteriorly as a small process, tip of prostomium terminates in retractile, muscular tip. Lateral

parts of peristomium clearly demarcated from prostomium, forming large peristomial umbrella. Peristomial umbrella carrying well-developed prototroch. Short palps developed in late larvae, attached on lateral-most parts of peristomium. Two pairs of red eyes arranged in an approximately straight line. Pharynx pigmented orange and the gut pigmented brown. Pygidium broad and spherical.

**Remarks.** Adult individuals of this species were collected from muddy sediments at 22 m depth in Onagawa Bay in December 2011 by using a Smith-McIntyre grab sampler. These adults were morphologically identified as *Scolelepis*, but they were not identified to species level as these specimens were all incomplete and in poor condition. As the 18S and 16S rRNA gene sequences obtained in the present study did not match any available *Scolelepis* sequences (Figs 2, 3), this species was referred to *Scolelepis* sp. 1. Planktonic larvae of this species were collected in Onagawa Bay in October during the study period. The larvae and adults were confirmed to match using molecular data (Fig. 3).

### *Scolelepis* sp. 2

Fig. 4L

**Larval morphology.** Thick and fusiform in overall shape. Prostomium bluntly pointed anteriorly, terminates in retractile, muscular tip. Lateral parts of peristomium clearly demarcated from prostomium, forming large peristomial umbrella. Peristomial umbrella carrying well-developed prototroch. Short palps developed in late larvae, attached on lateral-most parts of peristomium. Two pairs of red eyes arranged in an approximately straight line. Pharynx widely pigmented orange and the gut pigmented brown. Pygidium broad and spherical shaped.

**Remarks.** No adult individuals of this species were collected in the present study. Even though the 18S and 16S rRNA gene sequences obtained from larvae in the present study did not match any available *Scolelepis* sequences, this species was referred to *Scolelepis* sp. 2 as the larvae constitute a monophyletic clade with the other *Scolelepis* species with robust statistical support (Figs 2, 3).

Planktonic larvae of this species were found in Onagawa Bay in September. The larvae of this species are quite similar to those of *Scolelepis* sp. 1; however, the prostomium is more broadly pointed anteriorly and the gut diverticula are more strongly segmented in the former species.

### Genus *Spiophanes* Grube, 1860

**Larval diagnosis.** Overall shape slender. Prostomium small or broad, rounded or slightly notched anteriorly. Lateral parts of the peristomium slightly or moderately demarcated from the prostomium, palps on lateral-most parts of peristomium. Two pairs of red eyes present. In late larvae, a pair of prominent or small antero-lateral processes on prosto-

mium are often developed. Melanophore absent, some pigmentation patches of various colors are present on pharynx, proctodaeum, prostomium, peristomium, pygidium, and/or various locations of the body in late larvae. Nototrochs occur in all chaetigers from chaetigers II–IV onwards. Gastrotrochs occur in all chaetigers from chaetiger II onwards (Thorson 1946; Hannerz 1956; Carrasco 1976; Plate and Husemann 1994; Blake 2006).

## Identification key to species of the larvae belonging to the genus *Spiophanes* in northeastern Japan

1 Prostomium small; pharynx and proctodaeum colored in brown; yellow or yellow-brown pigments on prostomium, peristomium, and pygidium; a pair of small lateral processes on the prostomium developed in late larvae; small red pigment spots present on lateral part of posterior chaetigers..... ..... ***Spiophanes uschakowi***

– Prostomium broad; pharynx and proctodaeum colored in black; a pair of prominent lateral processes on the prostomium developed in late larvae; small red pigment spots absent..... ***Spiophanes* aff. *uschakowi***

### *Spiophanes uschakowi* Zachs, 1933

Fig. 4M, N

**Larval morphology.** Overall shape slender. Prostomium small and rounded anteriorly. Two pairs of red or dark red eyes present, lateral pair situated anteriorly. Late larvae bear very small antero-lateral processes on prostomium. Lateral parts of peristomium slightly demarcated from prostomium, palps attached on lateral-most parts of peristomium. Nototrochs occur from chaetiger IV onwards (Fig. 4N). Yellow pigments on the prostomium and pygidium, intense yellow-brown pigment on peristomium, inside of pharynx, and pygidium. Small red pigment spots present on lateral part of body (Fig. 4N). Black pigment in pharynx and proctodaeum absent.

**Remarks.** No adult individuals of this species were collected in the present study. However, gene sequences obtained from larvae of this species were almost identical (18S: 1732/1732, 16S: 341/342 bp) to that of *S. uschakowi* (KM998760 and MG878915) from Russia (Radashevsky et al. 2020a); therefore, this species was referred to *S. uschakowi*. Imajima (1991b) recorded four *Spiophanes* species from Japan: *S. kroyeri* Grube, 1860 (as *S. kroeyeri*); *S. japonicum* Imajima, 1991; *S. bombyx* (Claparède, 1870); and *S. urceolata* Imajima, 1991. Then, Meißner and Hutchings (2003) synonymized *S. urceolata* with *S. wigleyi* Pettibone, 1962. Additionally, the specimens from Japan formerly identified as *S. bombyx* were morphologically reexamined and identified as *S.* cf. *uschakowi* by Meißner and Blank (2009). In the present study, the presence of *S. uschakowi* in Japan was further supported by molecular analysis.

Only a few larvae of this species were collected in Onagawa Bay in November 2011. The overall larval morphology of this species somewhat resembles that of *S. kroyeri* de-

scribed by Hannerz (1956) in the following aspects: prostomium is relatively small and anteriorly rounded, the peristomium is not quite sharply demarcated from the prostomium, nototrochs occur from chaetiger IV onwards, and the brown pigmentation is present on the pygidium and inside the pharynx. However, *S. kroyeri* lacks small red pigment spots on the lateral part of the body and lateral processes on the prostomium even in 22-chaetiger larvae.

### *Spiophanes* aff. *uschakowi* Zachs, 1933

Fig. 4O, P

**Larval morphology.** Overall shape slender. Prostomium broad and slightly notched anteriorly. In late larvae, a pair of prominent antero-lateral processes on prostomium developed. Two pairs of red or dark red eyes present, lateral ones situated somewhat anteriorly. Lateral parts of peristomium moderately demarcated from prostomium, palps on lateral-most parts of peristomium. Nototrochs occur from chaetiger II onwards. Pharynx and proctodaeum black in color internally. Pygidium acquires dorsal cirri in late larvae. Some brownish, yellowish, or greenish pigmentation occurred on various locations of body in late larvae.

**Remarks.** Adult individuals of this species were collected from muddy bottom sediments at 22 m depth in Onagawa Bay in April and May 2012 by using a Smith-McIntyre grab sampler and from bottom sediments of the shallow subtidal zone in Sasuhama in February 2012. Adult morphology agrees with the description of *S.* cf. *uschakowi* by Meißner and Blank (2009) as well as with that of *S. bombyx* by Imajima (1991b). However, the 18S and 16S rRNA gene sequences of this species did not match those of *S. uschakowi* obtained from DDBJ/EMBL/GenBank (KM998760): there was a 0.29% (5/1750 bp) and 0.88% (3/342) difference, respectively between these two species. Therefore, this species was referred to *S.* aff. *uschakowi*. The larvae and adults were confirmed to 100% match using molecular data (Fig. 2).

Planktonic larvae of this species were collected in Onagawa Bay in November 2011 and in Sasuhama in February 2012. The larval morphology of *S.* aff. *uschakowi* was different from that of *S. uschakowi* in the following aspects: prostomium of the former is broad and slightly notched anteriorly, whereas that of the latter is relatively small and anteriorly rounded; the peristomium of the former is well demarcated from the prostomium, but that of the latter is relatively less demarcated; nototrochs of the former occur from chaetiger II onwards, whereas those of the latter occur from chaetiger IV onwards; pigmentation inside the pharynx is black in the former but brown in the latter; and black pigmentation in the proctodaeum is present in the former but absent in the latter. Black pigmentation in the pharynx and proctodaeum were also reported in the larvae of *S. bombyx* (Hannerz 1956), *S.* cf. *bombyx* (Blake 2006), and *S. duplex* (Chamberlin, 1919) (Blake 2006). However, the illustrations of *S.* cf. *bombyx* provided by Blake (2006: fig. 13.10C, D) are seemingly more similar to larvae of *Rhynchospio* than to those of *Spiophanes*.

## Subfamily SPIONINAE Söderström, 1920

### Genus *Boccardia* Carazzi, 1893

**Larval diagnosis.** Overall shape thick or slender and fusiform. Prostomium small or broad and rounded anteriorly. Three pairs of black eyes present, most lateral often double-eyes. Dorsal pigment pattern consists of single row of branching melanophores in most species, some species lack distinct dorsal melanophore. Lateral pigments present or absent. Ventral pigments absent. Nototrochs occur in all chaetigers except first two chaetigers. Gastrotrochs occur in irregular pattern. Modified chaetae develop in chaetiger V in late larvae (Söderström 1920, as *Polydora natrix*; Hartman 1941; Carrasco 1976; Woodwick 1977; Blake and Kudenov 1981; Duchêne 1984, 1989; Guérin 1991; Gibson 1997; Blake and Arnofsky 1999; Gibson and Smith 2004; Blake 2006; Kamel et al. 2010; Oyarzun and Brante 2015; Blake 2017).

#### Identification key to species of the larvae belonging to the genus *Boccardia* in northeastern Japan

1 Distinct dorsal melanophore absent; faint yellow coloration present on all over body ..... ***Boccardia pseudonatrix***
– Mid-dorsal melanophores arranged in a single row ..... **2**
2 Dorso-lateral spots of black pigment absent; overall body shape thick and fusiform; pharynx pigmented with black..... ***Boccardia* sp. 2**
– Dorso-lateral spots of black pigment present; overall body shape slender and fusiform; black pigment at pharynx present or absent ..... **3**
3 A prominent row of mid-dorsal melanophores from chaetiger III; dorso-lateral spots of black pigment present on chaetigers VII and VIII; black pigment in pharynx absent ..... ***Boccardia proboscidea***
– A prominent row of mid-dorsal melanophores from chaetiger IV; dorso-lateral spots of black pigment present from chaetiger V onwards; black pigment in pharynx present ..... ***Boccardia* sp. 1**

#### *Boccardia proboscidea* Hartman, 1940

Fig. 7A, B

**Larval morphology.** Slender and fusiform in overall shape, widest in middle of body. Prostomium rounded and slightly notched anteriorly. Three pairs of eyes present, most median pair rounded, lateral pairs double-eyes. Body entirely faint green in color. A prominent row of dorsal melanophores occurs medially from chaetiger III, lateral black pigment spots present on chaetigers VII and VIII in late larvae (Fig. 7B). Pygidium has dorsal gap, pigmented with weak dark color. Internally, vestibule light brown, gut either yellow or brown. Gastrotrochs on chaetigers V and VII.

**Remarks.** Adults of this species were non-boring and collected from mud deposits in crevices of shells of living *Crassostrea gigas* (Thunberg, 1793) (recently assigned to *Magallana*: see Backeljau 2018) oysters in Sasuhama in May 2011 and February 2016. Adult morphology agrees with the description of *B. proboscidea* by Sato-Okoshi (2000). The 18S and 16S rRNA gene sequences obtained in the present study match (18S: 1748/1748, 16S: 435/435 bp) that of *B. proboscidea* from USA (KJ546254) reported by Radashevsky et al. (2014) (Fig. 3). Therefore, this species was referred to *B. proboscidea*. The larvae and adults were confirmed to match (18S: 1768/1768, 16S: 472/472 bp) using molecular data (Fig. 2).

Planktonic larvae of this species were rare, and only one 15-chaetiger larva (Fig. 7B) was collected in Sasuhama in May 2011. Another 9-chaetiger larvae, which accidentally hatched from its egg capsule in an adult tube during the process of extraction of the adult specimens (Fig. 7A), was also collected on the same date. *Boccardia proboscidea* has been reported to have poecilogonous development (Gibson 1997; Oyarzun et al. 2011). However, Sato-Okoshi (2000) reported that Japanese populations only show lecithotrophic development, with no (or a very short) planktonic stage after hatching. The larval morphology of this species agrees with the description of that of *B. proboscidea* documented in Hartman (1941), Woodwick (1977), Blake and Kudenov (1981), Gibson (1997), Gibson and Smith (2004), Kamel et al. (2010), and Oyarzun and Brante (2015). The dorsal pigment pattern of these larvae resembles that of the larvae of *B. tricuspa* (Hartman, 1939) described by Carrasco (1976, as *B. proboscidea*; *fide* Blake and Kudenov 1978), *B. natrix* (Söderström, 1920) described by Söderström (1920, as *Polydora natrix*), and *B. columbiana* Berkeley, 1927 described by Blake and Arnofsky (1999) and Blake (2006) in having a single row of mid-dorsal melanophores. However, the dorsal pigment pattern of the larvae of *B. tricuspa* differs from that of *B. proboscidea* in having branching dorsal melanophores on chaetiger I and in lacking small black lateral pigment spots on chaetigers VII and VIII. The larvae of *B. natrix* also lack small black lateral pigment spots on chaetigers VII and VIII. *Boccardia columbiana* has extensively branching mid-dorsal melanophores from chaetiger II onward, whereas mid-dorsal melanophores are less branching and start from chaetiger III in *B. proboscidea*.

### *Boccardia pseudonatrix* Day, 1961

Fig. 7C

**Larval morphology.** Slender and fusiform in overall body shape. Prostomium rounded with a slight anterior notch. Three pairs of eyes present, most median pair rounded and lateral pairs double-eyes. Body entirely faint yellow. Dorsal melanophore absent, slight black pigmentation present (Fig. 7C, left). Pygidium with dorsal gap, pigmented with yellow. Internally, the vestibule and pharynx brown or black, gut green in color. Gastrotrochs on chaetigers V and VII.

**Remarks.** Adults of this species were non-boring and collected from mud deposits in crevices of shells of living *C. gigas* oysters in Tomiura. Adult morphology (see Abe

et al. 2019b) agrees with the descriptions of *B. pseudonatrix* from South Africa (Day 1967; Simon et al. 2010) and Australia (Sato-Okoshi et al. 2008, as *B. knoxi*; see Walker 2013). The 18S rRNA gene sequences obtained in the present study completely match (1714/1714 bp) that of *B. pseudonatrix* from South Africa (KY677895) reported by Williams et al. (2017) (Fig. 3). Therefore, this species was referred to *B. pseudonatrix*.

*Boccardia pseudonatrix* has been reported to have adelphophagic larvae with a short or absent planktonic phase (Sato-Okoshi et al. 2008; Simon 2015). The larvae herein reported accidentally hatched from egg capsules in an adult tube during the process of extraction of the adult specimens.

### *Boccardia* sp. 1

Fig. 7D–F

**Larval morphology.** Slender and slightly fusiform in overall shape, widest in anterior part of body. Prostomium rounded anteriorly. Three pairs of black eyes present, most median pair rounded and lateral pairs double-eyes. Body entirely yellowish in color. A prominent row of dorsal melanophores occurs medially from chaetiger IV, lateral black pigment spots present from chaetiger V onwards (Fig. 7E). Pygidium with dorsal gap, pigmented with black color. Vestibule black, gut orange in color internally. Larval chaetae on first chaetiger long especially in early larvae (Fig. 7D). Gastrotrochs on chaetigers III, V, VII, X, and XIII.

**Remarks.** No adult individuals of this species were collected in the present study. The 18S and 16S rRNA gene sequences obtained from the larvae did not match any available *Boccardia* sequences, but this species is very similar to the other *Boccardia* species in larval morphology and gene sequences (Figs 2, 3). Therefore, this species was referred to *Boccardia* sp. 1.

Planktonic larvae of this species were collected from Onagawa Bay in April, May, November, and December 2011, and January, February, March, and May 2012. The overall body shape of these larvae is slender and slightly fusiform, similar to those of *B. proboscidea*. However, other larval morphological characteristics differ between these two species: overall body color is faint yellow in the former species and faint green in the latter; the larval chaetae are longer in the former species than in the latter, especially in early larvae (Fig. 7D); lateral black spots are present on chaetiger V onwards in the former species but only on chaetigers VII and VIII in the latter species.

### *Boccardia* sp. 2

Fig. 7G

**Larval morphology.** Thick and fusiform in overall shape, widest at middle part of body. Prostomium extensively broad and anteriorly rounded. Three pairs of black eyes present, most median pair rounded and lateral pairs double-eyes. Body entirely faint


**Figure 7.** Light micrographs showing the morphologies of living spionid larvae of genera *Boccardia*, *Boccardiella*, and *Dipolydora* **A, B** *Boccardia proboscidea*, dorsal view of accidentally hatched 9-chaetiger (**A**) and 15-chaetiger larvae (**B**) **C** *Boccardia pseudonatrix*, dorsal (left) and ventral (right) view of accidentally hatched 10-chaetiger larvae **D–F** *Boccardia* sp. 1, dorsal view of 9-chaetiger (**D**) and dorsal (**E**) and ventral view (**F**) of 17-chaetiger larvae **G** *Boccardia* sp. 2, dorsal view of 15-chaetiger larva **H, I** *Boccardiella hamata*, dorsal view of 16-chaetiger (**H**) and 18-chaetiger larvae (**I**) **J, K** *Dipolydora bidentata*, dorsal view of 13-chaetiger (**J**) and 18-chaetiger larvae (**K**) **L** *Dipolydora* cf. *commensalis*, dorsal view of 21-chaetiger larva **M, N** *Dipolydora giardi*, dorsal view of 19-chaetiger (**M**) and lateral view of 21-chaetiger larvae (**N**) **O** *Dipolydora* cf. *socialis*, dorsal view of 18-chaetiger larva **P** *Dipolydora* sp., dorsal view of 7-chaetiger larva. Scale bars: 300 μm.

green in color in late larvae. A prominent row of dorsal ramified melanophores occurs medially from chaetiger IV onwards, lateral black pigment spots absent. Pygidium with dorsal gap, pigmented with weak dark color. Internally, vestibule black, gut orange in color. Gastrotrochs on chaetigers III, V, VII, X, and XIII.

**Remarks.** No adult individuals of this species were collected in the present study. The 18S and 16S rRNA gene sequences herein obtained from the larvae did not match any of the available *Boccardia* sequences, but this species is similar to the other *Boccardia* species in larval morphology and gene sequences (Figs 2, 3); therefore, this species was referred to *Boccardia* sp. 2.

Planktonic larvae of this species were collected from Onagawa Bay in December 2010 and November and December 2011, from Sasuhama in January 2013, and from Sendai Port in December 2010. The larval morphology of this species differs from that of other *Boccardia* larvae in having a thick and fusiform body shape.

### Genus *Boccardiella* Blake & Kudenov, 1978

**Larval diagnosis.** Overall shape thick and fusiform. Prostomium extensively broad and rounded anteriorly. Three pairs of black eyes present, most lateral pairs usually double-eyes. More than two pairs of dorsal melanophores from chaetiger III onwards. Lateral and ventral pigments present. Nototrochs occur in all chaetigers except first two. Gastrotrochs occur in irregular pattern. Modified chaetae develop in chaetiger V in late larvae (Rullier 1960, as *Polydora redeki*; Dean and Blake 1966, as *Boccardia*).

#### *Boccardiella hamata* (Webster, 1879)

Fig. 7H, I

**Larval morphology.** Thick and fusiform in overall shape, widest at middle part of body. Prostomium broad and anteriorly rounded, usually dusky brown anteriorly. Three pairs of black eyes present, most median pair rounded, lateral pairs usually double-eyes, occasionally divided into respective eyes. Black pigmentation usually presents ventrally on each lateral lip, occasionally absent. Dorsal pigmentation basically consists of a pair of medial bands, lateral branching melanophores, and small pigment patch at the base of notopodia in each chaetiger from chaetiger III onwards (Fig. 7H). These melanophores undergo expansion and contraction, sometimes coalescing to cover almost the whole of the dorsal surface as ramified pigmentation (Fig. 7I). Four transverse lines of black pigmentation sometimes fused as a single transverse band in chaetiger I. One or two pairs of lateral black pigmentation on chaetiger II. Two rows of band-shaped ventral pigmentation usually located on posterior edges of some chaetigers posterior to second chaetiger. A pair of black pigment patches on pygidium. Gastrotrochs on chaetigers III, V, VII, X, and XIII.

**Remarks.** Adults of this species were non-boring and collected from mud deposits in crevices of shells of living *C. gigas* oysters in Sasuhama in May 2011 and February 2016. Adult morphology agrees with the description of *B. hamata* by Sato-Okoshi (2000). Therefore, this species was referred to *B. hamata*. The larvae and adults were confirmed to match (18S: 1772/1772, 16S: 480/481 bp) using molecular data (Fig. 2).

Planktonic larvae of this species were frequently collected from Onagawa Bay, Gobu-ura, and Sasuhama in July and August. The larval morphology of this species agrees with that of *B. hamata* described by Dean and Blake (1966, as *Boccardia*).

### Genus *Dipolydora* Verrill, 1879

**Larval diagnosis.** Overall shape slender or slightly fusiform. Prostomium small rounded anteriorly. Three pairs of black eyes present, most lateral pairs often double-eyes. Ramified melanophore between central and lateral pairs of eyes usually absent, but present in some species (e.g., *D.* cf. *commensalis*). Dorsal pigment pattern consists of two rows of band or spot shaped melanophores or a transverse row of small melanophores at each chaetiger in most species, while some species have single row of branching mid-

dorsal melanophores (e.g., *D.* cf. *commensalis*) or completely lack melanophores (e.g., *D. armata*). Lateral and ventral pigments are present or absent. Nototrochs occur in all chaetigers except the first two chaetigers. Gastrotrochs occur in irregular pattern. Modified chaetae develop in chaetiger V in late larvae (Andrews 1891, as *Polydora*; Hannerz 1956, as *Polydora*; Hatfield 1965, as *Polydora*; Blake 1969, as *Polydora*; Carrasco 1976, as *Polydora*; Day and Blake 1979, as *Polydora*; Radashevsky 1989, as *Polydora*; Plate and Husemann 1994, as *Polydora*; Lewis 1998; Blake 2006; Blake 2017).

## Identification key to species of the larvae belonging to the genus *Dipolydora* in northeastern Japan

| | |
|---|---|
| 1 | Mid-dorsal single row of distinct melanophores present ..... ***Dipolydora* cf. *commensalis*** |
| – | Arrangement of dorsal melanophore otherwise ..... **2** |
| 2 | Black pigmentation on lateral peristomium present; a pair of band-shaped ventral black pigment present; notopodial lobes tipped with orange pigment in late larvae..... ***Dipolydora* cf. *socialis*** |
| – | Black pigmentation on lateral peristomium absent; ventral black pigment absent; notopodial lobes not tipped with orange pigment..... **3** |
| 3 | Some patchy black pigment between head and first chaetiger present ..... ***Dipolydora bidentata*** |
| – | Black pigment between head and first chaetiger absent ..... **4** |
| 4 | Two pairs of dorsal black pigment spots present; yellow-brown pigment on anterior margin of prostomium absent..... ***Dipolydora giardi*** |
| – | A pair of dorsal black pigment spots present; weak yellow-brown pigment on anterior margin of prostomium present ..... ***Dipolydora* sp.** |

### *Dipolydora bidentata* (Zachs, 1933)

Fig. 7J, K

**Larval morphology.** Overall shape elongated. Prostomium and pygidium small. Three pairs of black eyes present, most lateral pairs double-eyes. Black pigmentation patches on lateral peristomium absent. Some patchy black pigment occurs between head and first chaetiger. Two dorsal black bands begin on chaetiger II and continue to posterior end. Dorso-lateral pigment extend posteriorly along lateral side found on most chaetigers. Some black or brown pigment may occur on pygidium. Ventral pigment absent. Gastrotrochs on chaetigers V, VII, X, XIII, and XV.

**Remarks.** Adults of this species are shell-borers and were collected from shells of wild *C. gigas* oysters in Sasuhama in July 2012. Adult morphology agrees with the description of *D. bidentata* by Sato-Okoshi (1999). The 18S and 16S rRNA gene sequences obtained in the present study match (18S: 900/900, 16S: 473/475 bp) that of *D. bidentata* from Russia (JX228065) reported by Radashevsky and Pankova (2013) (Fig. 3). Planktonic larvae of this species were collected from Onagawa Bay in Novem-

ber 2011 and from Sasuhama in February 2012. The larvae and adults were confirmed to 100% match using molecular data (Fig. 2).

### *Dipolydora* cf. *commensalis* (Andrews, 1891)

Fig. 7L

**Larval morphology.** Overall shape elongated and slender. Prostomium small but wider than body and rounded anteriorly. Three pairs of eyes present, most lateral pairs double-eyes of kidney-shaped appearance. Ramified melanophores present around eyes. Black pigmentation on lateral peristomium absent. Median row of ramified melanophores from chaetiger I onwards. Lateral and ventral pigments absent. A central black pigment spot and a pair of dark brown pigments on pygidium. Pygidium has a dorsal notch and lacks appendages. Gastrotrochs on chaetigers III, V, VII, X, XIII, XV, XVII, XIX, XXI, and XXIII. Modified chaetae develop in chaetiger V in late larvae.

**Remarks.** No adults of this species were collected in the present study. The 18S and 16S rRNA gene sequences obtained from the larvae of this species neither match nor constitute a monophyletic clade with any of the other available spionid sequences (Figs 2, 3). However, this species was tentatively identified as *D.* cf. *commensalis* based on its larval morphology, as it includes the characteristic dorsal pigment pattern of larvae of *D. commensalis* as described by Andrews (1891), Hatfield (1965), Blake (1969), and Radashevsky (1989) (described as *Polydora commensalis* by all of these authors). The combination of a slender body and a single dorsal median row of distinct melanophores from chaetiger I to the end of the body is distinctive among spionid larvae and has not been reported for any other spionid species. Currently, there are no records of *D. commensalis* from Japan; however, the presence of this species in Japan is expected as it has been reported from the Asian continental coast of the Sea of Japan and the Kurile Islands (Radashevsky 1993). This species is an obligate symbiont of hermit crabs (Blake 1996; Williams and McDermott 1997), but little effort was devoted to collecting hermit crab shells in the present study.

Notably, the results of the phylogenetic analysis in the present study showed that *D.* cf. *commensalis* deviates from the monophyletic clade constituted by many other *Dipolydora* species. This result supports the suggestion by Blake (1971) that *D. commensalis* may represent a distinct genus as its morphology deviates widely from other species of the genus *Polydora* and *Dipolydora*.

Only three individuals of planktonic larvae of this species were collected in Sasuhama in January 2013. A small patch of lateral black pigments on the anterior margin of each chaetiger in late larvae was described in Hatfield (1965) and Blake (1969). However, these pigments were not observed in the present study, as in Andrews (1891) and Radashevsky (1989). Although Hatfield (1965) and Blake (1966) noted the high similarity between the larval morphologies of *D. commensalis* and *Polydora hermaphroditica* Hannerz, 1956, the adult morphologies of these two species were reported to be completely different (Bhaud 1966). The dorsal pigment pattern of *P. hermaphroditica* larvae reported by Hannerz (1956) rather resembles those of *Polydora glycymerica* and

*Polydora* cf. *glymymerica* larvae reported by Radashevsky (1989) and in the present study, respectively.

***Dipolydora giardi* (Mesnil, 1896)**
Fig. 7M, N

**Larval morphology.** Overall body shape elongated and slender. Prostomium small and rounded anteriorly. Three pairs of black eyes arranged in transverse row, most lateral pairs double-eyes. Black pigmentation on lateral peristomium absent. Two pairs of dorsal black spots begin on chaetiger III onwards and continue to posterior end, sometime medial pair in first 2–4 chaetigers band-shaped. Small medial spot of black pigment on posterior margin of each chaetiger usually from chaetigers III, rarely from V or VI, in late larvae. Two small spots of black pigmentation occur lateral to the medial black pigmentation from approximately chaetiger VI or VII. A small black pigment spot, not visible dorsally, present on antero-lateral edges from chaetiger II onwards. Black pigment occurs on pygidium. Rust-colored pigment occurs in pharynx. Ventral pigment absent. Some metamorphosing larvae reduce pigmentation over the entire body and present whitish appearance with eyes fused and appears as one pair (Fig. 7N). Gastrotrochs on chaetigers III, V, VII, X, XIII, and XV.

**Remarks.** Adults of this species are shell-borers and were collected from shells of cultured *Mizuhopecten yessoensis* (Jay, 1857) (formerly as *Patinopecten yessoensis*) scallops suspended in Onagawa Bay in December 2010. Adult morphology agrees with the description of *D. giardi* by Sato-Okoshi (1999). Therefore, this species was referred to *D. giardi*. The larvae and adults were confirmed to match using molecular data (Fig. 3).

The 18S rRNA gene sequences of this species are very similar to that of *D. capensis* 1PE from South Africa (KY677896) reported by Williams et al. (2017), but there is a slight difference between their sequences (0.12% difference: 2/1714 bp). It is unclear whether this difference indicates that these two are the same or different species because two different 18S rRNA gene sequences have been reported from South Africa and are currently under the same species name (*D. capensis*) (Table 2, Fig. 3). No gene sequences of *D.* cf. *giardi* previously recorded from South Africa (Simon 2011) are available.

Planktonic larvae of this species were collected from Onagawa Bay in December 2010, June, July, October, November, and December 2011, and December 2012, and from Sasuhama in January 2013. The larval morphology of this species was previously described from California by Day and Blake (1979, as *Polydora giardi*). The morphology and dorsal pigment pattern of late larvae described by these authors resembles that reported here, but there are slight differences: two golden pigment spots present on either side of chaetiger I in the former description but absent in the latter; two small lateral melanophores present on chaetigers I and II in the former description but absent in the latter; a medial black pigmentation beginning from chaetiger II onwards in the former description but from chaetiger III onwards in the latter description; and two small spots of black pigmentation lateral to the medial black pigmentation starting from chaetiger III in the former description but from more posteriorly in the latter

description. These differences between specimens from Japan and California may indicate that they are different species, or that intraspecific variation occurs in larval dorsal pigmentation. Day and Blake (1979) pointed out differences in reproductive traits between the Californian and French populations and suggested the existence of two different species. Therefore, more than one species may be included under the name of *D. giardi*, which currently is reported with a worldwide distribution (Radashevsky and Petersen 2005).

### *Dipolydora* cf. *socialis* (Schmarda, 1861)

Fig. 7O

**Larval morphology.** Late larvae usually thick and slightly fusiform in shape, although not as much as the larvae of *Boccardia* sp. 2 (Fig. 7G), *Boccardiella hamata* (Fig. 7H, I), and *Pseudopolydora* species (Fig. 9A–I). Anterior margin of prostomium has yellow-brown pigment. Three pairs of black eyes arranged in transverse row, most lateral pairs double-eyes. Band of black pigment on each lateral part of the peristomium. First dorsal black melanophores occur as paired bands on chaetiger III and continue through to chaetiger V. From chaetiger VI, two pairs of dorsal black spots or bands occur and continue to posterior end of body. From chaetiger IV or V and continuing posteriorly, clusters of small black pigmented cells present in transverse row on dorsal posterior half of chaetigers. Lateral pigment found on late larvae on chaetiger II. Each notopodial lobe tipped with orange pigment, small patch of black pigment at the base of notopodial lobes. Ventral pigment consists of paired bars on posterior border of chaetigers, commencing with chaetiger II. Some black or brown pigment may occur on pygidium. Gastrotrochs occur on chaetigers III, V, VII, X, XIII, XV and XVII.

**Remarks.** Adults of this species were non-boring and collected from muddy bottom sediment at 22 m depth in Onagawa Bay in December 2010 by using a Smith-McIntyre grab sampler and from bottom sediments of shallow subtidal zone in Sasuhama in April 2013. Adult morphology agrees with the description of *D. socialis* by Sato-Okoshi (2000). The 18S rRNA gene sequence obtained in the present study showed a 0.35% (6/1715 bp) difference with that of *D.* cf. *socialis* from South Africa (KY677899) reported by Williams et al. (2017), which may indicate that these two are different species. The 18S rRNA gene sequence obtained in the present study rather closer to that of *D. carunculata* (940/942 bp match) reported by Radashevsky and Pankova (2013), but the 16S gene sequence showed a 2.3% (11/475 bp) difference with that of *D. carunculata*. As described above, since the taxonomic status of the species reported here is uncertain, we tentatively referred to it as *D.* cf. *socialis*.

Planktonic larvae of this species were collected from Onagawa Bay in November 2010 and 2011, and in October 2012. The larvae and adults were confirmed to match (18S: 1770/1770, 16S: 473/475 bp) using molecular data (Fig. 2). The larval morphology of this species agrees with that of *D. socialis* described as *Polydora socialis* by Blake (1969) and Carrasco (1976).

***Dipolydora* sp.**
Fig. 7P

**Larval morphology.** Overall body shape slender. Prostomium small and rounded anteriorly. Anterior margin of prostomium has weak yellow-brown pigment. Three pairs of black eyes present in transverse row, most lateral pairs double-eyes. Black pigmentation on lateral peristomium absent. A pair of dorsal black spots present on chaetiger III onwards. A small medial spot of black pigment on posterior margin of chaetiger III. Some black pigment occurs on pygidium. Ventral pigment absent. Gastrotrochs occur on chaetigers III and V.

**Remarks.** No adult individuals of this species were collected in the present study. The 18S and 16S rRNA gene sequences obtained from larvae in the present study did not match any available *Dipolydora* sequences. As the larvae specimens formed a monophyletic clade with the other *Dipolydora* species (excluding *D. armata*, *D. capensis* 1GG, *D.* cf. *commensalis*, and *D. quadrilobata*) with robust statistical supports (Figs 2, 3), this species was referred to *Dipolydora* sp.

**Genus *Polydora* Bosc, 1802**

**Larval diagnosis.** Overall shape slender or slightly fusiform. Prostomium broad or small and rounded anteriorly. Three pairs of black eyes present, most lateral pairs often double-eyes. Some species have ramified melanophore between central and lateral pairs of eyes. Dorsal pigmentation usually consists of two rows of bands, spots, or branching melanophores in most species, while some species have a single row of mid-dorsal melanophores (e.g., *Polydora* cf. *glycymerica*). Lateral and ventral pigments present or absent. Nototrochs occur in all chaetigers except first two. Gastrotrochs occur in irregular pattern. Modified chaetae develop on chaetiger V in late larvae (Wilson 1928; Thorson 1946; Hannerz 1956; Hopkins 1958; Woodwick 1960; Blake 1969; Carrasco 1976; Radashevsky 1986, 1988, 1989; 1994, 2005; Plate and Husemann 1994; Sato-Okoshi 1994; Williams 2001; Radashevsky and Cárdenas 2004; Blake 2006; Radashevsky et al. 2006; Zhang et al. 2009; Gao et al. 2011; David et al. 2014; Barros et al. 2017; Blake 2017; Radashevsky and Migotto 2017; Ye et al. 2017).

## Identification key to species of the larvae belonging to the genus *Polydora* in northeastern Japan

| | | |
|---|---|---|
| 1 | Mid-dorsal single row of branching melanophores present | ***Polydora* cf. *glycymerica*** |
| – | Mid-dorsal single row of branching melanophores absent | **2** |
| 2 | Vestibule and pharynx with black pigmentation | ***Polydora brevipalpa*** |
| – | Vestibule and pharynx not pigmented with black | **3** |
| 3 | Dorsal melanophores on each chaetiger faint | ***Polydora* sp. 2** |
| – | Dorsal melanophores on each chaetiger distinct | **4** |

4 Black or brown pigmentation on lateral part of peristomium present ..... **5**
– Black or brown pigmentation on lateral part of peristomium absent ..... **8**
5 Distinct ventral pigment spot (yellow-green, brown, or black) present ..... ***Polydora cornuta***
– Distinct ventral pigment spot absent ..... **6**
6 Black pigmentation on lateral part of lateral peristomium present ..... ***Polydora* sp. 3**
– Brown pigmentation on lateral part of lateral peristomium present ..... **7**
7 Two rows of dorsal melanophores from chaetigers III–VI or VII band-shaped, followed by large branching melanophores in posterior chaetiger ..... ***Polydora onagawaensis***
– Two rows of dorsal melanophores on anterior chaetiger dot-like or short band-shaped, followed by dot-like not branching melanophores in posterior chaetiger ..... ***Polydora* sp. 1**
8 Two rows of dorsal melanophores mostly band-shaped with some of them slightly branching ..... ***Polydora* cf. *spongicola***
– Two rows of dorsal melanophores band-shaped in anterior chaetigers, followed by branching melanophores in posterior chaetiger ..... **9**
9 Two rows of dorsal melanophores from chaetigers III–VII band-shaped, followed by pairs of large branching melanophores ..... ***Polydora hoplura***
– Two rows of faint dorsal melanophores from chaetigers II–V or VII band-shaped, followed by pairs of branching melanophores in posterior chaetigers or whole of dorsal surface covered by finely ramified black pigmentation ..... ***Polydora* sp. 2**

### *Polydora brevipalpa* Zachs, 1933

Fig. 8A, B

**Larval morphology.** Overall shape slender and slightly fusiform. Prostomium broad and rounded anteriorly. Three pairs of black eyes present, innermost pair rounded, lateral pairs double-eyes, ramified melanophore between innermost and lateral two pairs of eyes usually present. Black pigment on lateral peristomium absent. Dorsal pigmentation consists of two rows of melanophores from chaetiger III. Dorsal melanophores undergo expansion and contraction, may expand to branching melanophores or ramified appearance or covered almost whole of dorsal surface by very finely ramified black pigments (Fig. 8B), or they contract to dot-like pigmentation patches (Fig. 8A). Lateral and ventral pigments absent. Vestibule and pharynx pigmented with black, gut pigmented with orange color. Modified chaetae develop on chaetiger V in late larvae. Gastrotrochs occur on chaetigers III, V, VII, X, XIII, XV, and XVII.

**Remarks.** Adults of this species are boring and were collected from shells of cultured *M. yessoensis* scallops suspended in Onagawa Bay in February 2011 and Mutsu Bay in October 2011. This species was identified as *P. brevipalpa* as adult morphology

agrees with the descriptions by Sato-Okoshi (1999) and Sato-Okoshi and Abe (2012). The larvae and adults were confirmed to 100% match using molecular data (Fig. 2).

Planktonic larvae of this species were collected from Onagawa Bay in April, May, and July 2011 and from Sasuhama in April 2011. The pair of large ramified or dot-like melanophores from chaetiger III distinguishes larvae of this species from those of other *Polydora* species. Blake (2017) reported similar dorsal pigment patterns in the larvae of *Polydora spongicola* Berkeley & Berkeley, 1950. However, the larvae of *P. spongicola* in Blake (2017) differ from those of *P. brevipalpa* in having dorsal melanophores from chaetiger II instead of chaetiger III, dark green colored intestine instead of orange, and non-pigmented pharynx instead of pigmented with black. Reproduction and life history of this species was reported in Sato-Okoshi et al. (1990) and Sato-Okoshi (1994) (both as *P. variegata*).

### ***Polydora cornuta* Bosc, 1802**

Fig. 8C, D

**Larval morphology.** Overall shape slender. Prostomium broad and rounded anteriorly. Three pairs of black eyes present, median pair rounded, most lateral pairs double-eyes, ramified melanophores between first median and the second lateral pair of eyes usually present. In late larval stage, anterior part of prostomium and lateral lips of peristomium pigmented yellow or brown. Small spots of black pigments occur on lateral parts of peristomium. Dorsal pigmentation consists of two rows of melanophores from chaetiger III with those of anterior four chaetigers band-shaped and then replaced by rounded or ramified melanophores from chaetiger VII onwards. Three rows of small faint dorsal spots of brown pigment present on posterior edge from chaetigers III or IV onwards in late larvae. Lateral pigment on chaetigers II, III, and often VI–XI extensive compared to that on other chaetigers. Large yellow or brown chromatophores occur ventrally from chaetigers V or VI onwards, usually three chromatophores arranged in transverse line except on gastrotroch-bearing chaetigers where single midventral chromatophores present. Black pigment spots occur on ventral side of body (Fig. 8D) and mid-dorsal part on pygidium (Fig. 8C). Gastrotrochs occur on chaetigers III, V, VII, X, XIII, XV and XVII.

**Remarks.** Adults of this species were non-boring and collected from mud deposits in crevices of shells of living *C. gigas* oysters in Sasuhama in June 2011 and from intertidal bottom sediment in Gamo Lagoon in August 2012. This species was identified as *P. cornuta* as adult morphology agrees with the description by Sato-Okoshi (2000) and Radashevsky (2005). The larvae and adults were confirmed to match (18S: 1770/1770, 16S: 468/470 bp) using molecular data (Fig. 2).

Rice et al. (2008) suggested that at least three sibling species may be involved in North America under the name of *P. cornuta* by differences of mitochondrial COI sequences between California, Florida, and Maine populations. Takata et al. (2011) reported that the *P. cornuta* from Fukuyama in the Seto Inland Sea, western Japan is

genetically close with the California/New Zealand lineage. It is unclear to which lineage the eastern Japan populations belong. The 18S rRNA gene sequence obtained in the present study showed a 1.9% (5/421 bp) difference with that of *P. cornuta* from Netherlands (KC686637).

Planktonic larvae of this species were collected from Gamo Lagoon in August 2012. The larval morphology of this species generally agrees with the descriptions of *P. cornuta* by Hannerz (1956, as *P. ligni*), Blake (1969, as *P. ligni*), Plate and Husemann (1994, as *P. ligni*), and Radashevsky (2005). Peristomial melanophores, which were reported by Hannerz (1956) and Blake (1969) but not by Radashevsky (2005), and middorsal vesiculate melanophores, which were reported by Radashevsky (2005) but not described by Hannerz (1956) and Blake (1969), were both present in specimens of the present study. Ventral pigmentation pattern was consistent with the description by Blake (1969) and Radashevsky (2005) instead of Hannerz's (1956) description. The larval dorsal pigmentation pattern, similar to that of *P. cornuta*, is typically found in many other *Polydora* species. This species can, however, be distinguished by the characteristic ventral yellow pigmentation pattern as the yellow pigment on the ventral side of the other *Polydora* species is diffusely scattered and does not appear regularly arranged when present (Radashevsky 2005).

### *Polydora* cf. *glycymerica* Radashevsky, 1993

Fig. 8E

**Larval morphology.** Overall shape elongated and slender. Prostomium small and rounded anteriorly. Three pairs of black eyes present, most lateral pairs double-eyes. Ramified melanophores between middle and lateral pair of eyes absent. Pigmentation on lateral peristomium absent. Two rows of ramified melanophores on chaetigers III–VI, and a median row of ramified melanophores from chaetiger VII onwards. Lateral and ventral pigments absent. A pair of black pigments occur on pygidium. Pygidium has a dorsal notch and lacks appendages. Gastrotrochs absent in 25-chaetiger larvae, probably already lost. Modified chaetae develop on chaetiger V.

**Remarks.** No adult individuals of this species were collected in the present study. The 18S and 16S rRNA gene sequences obtained from larvae in the present study did not match any of the available *Polydora* sequences. However, as the larvae formed a robustly supported clade with other *Polydora* species (Figs 2, 3), this species was referred to as the genus *Polydora*. Furthermore, the larval morphology including the characteristic dorsal pigment pattern of this larvae matches that of the larvae of *P. glycymerica* described by Radashevsky (1989). Therefore, this larva was tentatively identified as *P.* cf. *glycymerica*. However, there were slight differences between the present specimens and Radashevsky (1989) description: the two rows of ramified melanophores continued until chaetiger VI in the present description, whereas it continues to chaetigers VII–X according to Radashevsky (1989); ramified melanophores between the middle and lateral pair of eyes are present in the former description while absent in the latter; larvae of


**Figure 8.** Light micrographs showing the morphologies of living spionid larvae of the genus *Polydora* **A, B** *Polydora brevipalpa*, dorsal view of 15-chaetiger (**A**) and 17-chaetiger larvae (**B**) **C, D** *Polydora cornuta*, dorsolateral view of 11-chaetiger larva (**C**) and ventral view of 17-chaetiger larva (**D**) **E** *Polydora* cf. *glycymerica*, dorsal view of 25-chaetiger larva **F** *Polydora hoplura*, dorsal view of 15-chaetiger larva **G–I** *Polydora onagawaensis*, dorsal view of 10-chaetiger (**G**) and 18-chaetiger larvae (**H**), and lateral view of 16-chaetiger larva (**I**) **J** *Polydora* cf. *spongicola*, dorsal view of 17-chaetiger larva **K, L** *Polydora* sp. 1, dorsal view of 7-chaetiger (**K**) and 16-chaetiger larvae (**L**) **M** *Polydora* sp. 2, dorsal view of 23-chaetiger larva **N–P** *Polydora* sp. 3, dorsal view of 13-chaetiger (**N**) and dorsal (**O**) and lateral view (**P**) of 18-chaetiger larvae. Scale bars: 300 μm.

*P.* cf. *glycymerica* collected in the present study were 25-chaetigers with > 2.0 mm long (Fig. 8E), whereas the largest larva observed by Radashevsky (1989) was a 20-chaetiger specimen 1.8 mm long. Further studies should test whether these differences are attributable to individual or developmental variabilities or interspecific differences.

The dorsal median single row of ramified melanophores is distinct in the larvae of the genus *Polydora*. The larvae of *Polydora hermaphroditica* also have a dorsal median row of ramified melanophores such as that of the larvae of *P.* cf. *glycymerica* and *P. glycymerica* (Hannerz 1956; Plate and Husemann 1994). However, the first species differs from the other two by the absence of two rows of ramified melanophores on anterior chaetigers.

Only one individual of planktonic larva of *P.* cf. *glycymerica* was collected in Onagawa Bay in October 2011. *Polydora glycymerica* was previously recorded as a shell-borer of *Macridiscus aequilatera* (G. B. Sowerby I, 1825) from Oarai, Japan (Sato-Okoshi 1999).

***Polydora hoplura*** **Claparède, 1868**
Fig. 8F

**Larval morphology.** Overall body shape slender or somewhat fusiform. Prostomium broad and rounded anteriorly. Three pairs of black eyes present, most lateral pairs double-eyes. Ramified melanophores between first and second innermost pair of eyes absent. Black pigmentation patches on lateral peristomium absent. Dorsal pigmentation consists of two rows of melanophores from chaetiger III with those of first five pairs band-shaped and then replaced by ramified melanophores in posterior chaetigers. Lateral pigments found on late larvae on chaetigers II–IV. Dorsolateral pigments at base of the parapodia start from chaetiger VII. A pair of black pigment occur on pygidium. Ventral pigment absent. Modified chaetae develop in chaetiger V in late larvae. Gastrotrochs occur on chaetigers III, V, VII, X, XIII, and XV.

**Remarks.** This species is a shell-borer, and adult specimens were collected from the turban snail *Omphalius rusticus* (Gmelin, 1791) in Gobu-ura and Onagawa Bay. This species was identified as *P. hoplura* as its adult morphology agrees with descriptions by Sato-Okoshi and Abe (2012, as *P. uncinata*) and Sato-Okoshi et al. (2017). The larvae and adults were confirmed to match (18S: 1769/1769, 16S: 464/475 bp) using molecular data (Fig. 2).

Only late larvae were found in July in Onagawa Bay. The larval morphology of this species agrees with descriptions by Wilson (1928) and Radashevsky and Migotto (2017). This species has adelphophagic and lecithotrophic larval development, in which larvae feed on nurse eggs in brood capsules, hatch at a very late stage, and have only a short pelagic life (Wilson 1928; Read 1975; Sato-Okoshi et al. 2008, as *P. uncinata*; Radashevsky and Migotto 2017). The poecilogenous development of this species with planktotrophic and adelphophagic planktonic larvae was reported by David et al. (2014), David and Simon (2014), and Simon (2015).

***Polydora onagawaensis*** **Teramoto, Sato-Okoshi, Abe, Nishitani & Endo, 2013**
Fig. 8G–I

**Larval morphology.** Overall body shape slender. Prostomium slightly broad and rounded anteriorly. Three pairs of black eyes present; median pair of eyes rounded, most lateral pairs double-eyes. Ramified melanophore between middle and lateral pair of eyes usually present (Fig. 8G, H). Weak brown pigmentation located on lateral parts of peristomium, behind prototroch, occasionally much paler or absent. Dorsal pigmentation consists of two rows of melanophores from chaetiger III with those of first IV–VI band-shaped and subsequently replaced by ramified melanophores. These melanophores undergo expansion and contraction. Lateral pigment found on chaetigers II and III in late larvae (Fig. 3G). Dorsolateral pigment at base of most parapodia, often appears to coalesce with dorsal pigment bands on anterior part of body (Fig. 8H). Pygidium has a dorsal notch and lacks appendages; a pair of black pigment patches occur on pygidium. Ventral brown pigment may be present on posterior part of late larvae

(Fig. 8I). Telotroch well developed. Gastrotrochs on chaetigers III, V, VII, X, XIII, and XV; those of chaetigers III and V lost in late larvae (Fig. 8I). In late larvae, modified chaetae develop in chaetiger V.

**Remarks.** This species is a shell-borer, and adult individuals were collected from shells of the wild turban snail *O. rusticus*, cultured scallop *M. yessoensis*, and wild and cultured *C. gigas* oysters in Onagawa Bay and Sasuhama, northeastern Japan. This species was identified as *P. onagawaensis* as adult morphology agrees with the description by Teramoto et al. (2013). The larvae and adults were confirmed to match (18S: 1771/1771, 16S: 472/473 bp) using molecular data (Fig. 2).

Planktonic larvae of this species were abundant from November to June in Onagawa Bay during the study period. The larval morphology of this species is similar to that of *Polydora* sp. 3 (see below). However, the former species has weak brown pigmentation on the lateral parts of the peristomium, whereas the latter species has large patches of black pigment on this region.

### *Polydora* cf. *spongicola* Berkley & Berkeley, 1950

Fig. 8J

**Larval morphology.** Overall body shape slender and slightly fusiform. Prostomium broad and rounded anteriorly. Three pairs of black eyes present; median eyes rounded, most lateral pairs double-eyes. Ramified melanophores between middle and lateral pair of eyes absent. Black pigment on lateral peristomium absent. Dorsal pigmentation consists of two rows of band-shaped melanophores from chaetiger II. These melanophores undergo expansion and contraction, expand to ramified melanophores or contract to non-ramified band-shaped melanophores. Lateral and ventral pigments absent. In late larvae modified chaetae develop in chaetiger V. Gastrotrochs on chaetigers III, V, VII, X, XIII, and XV.

**Remarks.** Adults of this species were collected from mud tubes constructed on the sponge *Mycale* sp. in Moroiso Bay, Misaki Peninsula (Table 1). The morphology of its modified spines in chaetiger V and the sponge-associated ecology of adults match the description of *P. spongicola* by Radashevsky (1993). However, this species was referred to *P.* cf. *spongicola* because the adult specimens were in poor condition, which hindered their morphology examination. The larvae and adults were confirmed to match (18S: 1770/1771, 16S: 474/475 bp) using molecular data (Fig. 2).

Only one planktonic larva of this species was collected in Sasuhama in January 2013. The larval morphology of *P.* cf. *spongicola* closely resembles that of *P. spongicola* described by Radashevsky (1988, as *Polydora uschakovi* Buzhinskaja, 1971) from Russia. *Polydora uschakovi* originally described from Russia was synonymized with *P. spongicola* (type locality: Canada) by Radashevsky (1993). Later, Blake (2017) described the larvae of *P. spongicola* from California and doubted this synonymization because, despite the similarities between the larvae from Russian and California, there are several morphological differences including the nature of the major spines of chaetiger V and the distribution of nototrochs and gastrotrichs. However, the larval dorsal pigment

pattern of *P. spongicola* described by Blake (2017) greatly differs from those of *P.* cf. *spongicola* in the present study and of *P. spongicola* in Radashevsky (1988) but resembles that of *P. brevipalpa* in the present study. Conspecificity between *P. uschakovi* and *P. spongicola* should be verified in future studies.

### *Polydora* sp. 1

Fig. 8K, L

**Larval morphology.** Overall body shape slender. Prostomium broad and rounded anteriorly. Three pairs of black eyes present; median eyes rounded and lateral pairs double-eyes. Ramified melanophore between innermost and next to innermost pairs of eyes absent. Weak brown pigmentation on lateral parts of peristomium present or absent. Dorsal pigmentation consists of two rows of melanophores from chaetiger III, those of first five pairs band-shaped and remaining pairs dot-like in late larvae (Fig. 8L). These melanophores all dot-like in early larvae (Fig. 8K). Lateral pigment found on chaetigers II, IX, X, and XI in late larvae. Dorsolateral pigment at base of parapodia on posterior chaetigers. A pair of black and brown pigment patches occur on pygidium. Ventral brown pigment present on posterior part of late larvae. Pygidium has a dorsal notch and lacks appendages. Telotroch well developed. In late larvae, modified chaetae develop in 5$^{th}$ chaetiger.

**Remarks.** Adults of this species are shell-borer and were collected from the shell of the turban snail *O. rusticus* in Sasuhama. The adults of this species have characteristic conspicuous black bars in their palps and are morphologically similar to *Polydora neocaeca* Williams & Radashevsky, 1999. *Polydora haswelli* previously recorded in Japan (Sato-Okoshi and Abe 2013) was reexamined as *P. neocaeca* by comparing morphology and molecular sequences with the specimens from near the type locality (Malan et al. 2020). As the 18S and 16S rRNA gene sequences of *Polydora* sp. 1 and *P. neocaeca* showed differences (18S: 8/1771, 16S: 40/476 bp), the specimens collected in the present study were referred to a different species. Only two individuals of planktonic larvae of this species were collected in Onagawa Bay in April and July 2011. The larvae and adults were confirmed to 100% match using molecular data (Fig. 2).

### *Polydora* sp. 2

Fig. 8M

**Larval morphology.** Overall body shape slender. Prostomium broad and rounded anteriorly. Three pairs of black eyes present, most lateral pairs double-eyes. Ramified melanophores between first and second innermost pairs of eyes absent. Pigmentation on lateral peristomium weak brown or absent. Dorsal pigmentation consists of two rows of melanophores from chaetiger II, with those of first 4–6 chaetigers being band-shaped and then replaced by ramified melanophores in posterior chaetigers. Dorsal pigments faint, undergo expansion and contraction, expand to cover almost whole of

dorsal surface as finely ramified black pigmentation (Fig. 8M) or contract to band-shaped or dot-like black pigments without ramification. Faint lateral pigment found on late larvae on chaetigers VII onwards. Ventral pigments absent. A pair of brown pigments occur on the pygidium. Pygidium has a dorsal notch and lacks appendages. Gastrotrochs on chaetigers III, V, VII, X, XIII, XV, XVII, and XIX. Modified chaetae develop in chaetiger V in late larvae.

**Remarks.** No benthic adult stages were collected in the present study. These larvae formed a robustly supported monophyletic clade with other *Polydora* species (Figs 2, 3). Nevertheless, this species was identified as a member of *Polydora*. As the 18S and 16S rRNA gene sequences obtained from the larvae did not match any other available *Polydora* sequences, this species was referred to *Polydora* sp. 2.

Only two individuals of planktonic larvae of this species were collected from Sasuhama and Gamo Lagoon in January 2013. The faint dorsal pigmentation of the larvae of this species is unique among the members of *Polydora* collected in the present study.

### *Polydora* sp. 3

Fig. 8N–P

**Larval morphology.** Overall body shape slender. Prostomium broad and rounded anteriorly. Three pairs of black eyes present; median pair of eyes rounded, most lateral pairs double-eyes, ramified melanophore between innermost and next to innermost pairs of eyes present. Large patches of black pigment located on lateral part of peristomium, behind prototroch. Dorsal pigment pattern consists of two rows of melanophores from chaetiger III with those of first four or five chaetigers being band-shaped and then replaced by ramified branching melanophores (Fig. 8O). These melanophores undergo expansion and contraction. Lateral pigment found on chaetigers II–IV, resumes again from chaetiger VII in late larvae (Fig. 8P). A pair of black pigment patches occur on pygidium. Ventral brown and black pigment present on posterior part in late larvae ready to metamorphose. Pygidium has a dorsal notch and lacks appendages. Telotroch well developed. Gastrotrochs on chaetigers III, V, VII, IX, X, XIII, XV, and XVII, lost on chaetigers III and V in late larvae (Fig. 8P). In late larvae, modified chaetae develop in chaetiger V.

**Remarks.** No benthic adult stages were collected in the present study. The 18S rRNA gene sequences obtained from the larvae did not match any available *Polydora* sequences. As the larvae formed a robustly supported monophyletic clade with other *Polydora* species (Figs 2, 3), this species was referred to *Polydora* sp. 3.

Planktonic larvae of this species were collected from December to June in Onagawa Bay every year during the study period. Planktonic larvae of this species were previously reported to be abundant in Onagawa Bay in the winter season from December to March (Abe et al. 2014, as *Polydora* sp.). Large patches of black pigment on the lateral peristomium are the main characteristic of this species and differentiate it from the other species of the genus observed in the present study, even at early planktonic stages (Fig. 8N).

### Genus *Pseudopolydora* Czerniavsky, 1881

**Larval diagnosis.** Overall body shape thick and fusiform. Prostomium broad and rounded or gently notched anteriorly. Three pairs of black eyes present, most lateral often double-eyes. Mid-dorsal melanophore on the first chaetiger present in many species, absent in some species. Dorsal pigmentation consists of one or two pairs of branching melanophores (except *P. rosebelae*: mid-dorsal single row of melanophores present). Lateral and ventral pigments present or absent. Nototrochs occur in all chaetigers except first two chaetigers. Gastrotrochs occur in irregular pattern. Modified chaetae in chaetiger V and ventral hooded hooks from chaetiger VIII onwards develop in late larvae (Hannerz 1956, as *Polydora*; Rullier 1963, as *Polydora*; Rasmussen 1973; Blake and Woodwick 1975; Srikrishnadhas and Ramamoorthi 1977; Wu and Chen 1980; Radashevsky 1983, 1985; Plate and Husemann 1994, as *Polydora*; Hsieh 1994; Blake 2006; Radashevsky and Migotto 2009; Kondoh et al. 2017).

#### Identification key to species of the larvae belonging to the genus *Pseudopolydora* in northeastern Japan

| | |
|---|---|
| 1 | A pair of dorsal melanophores on each chaetigers.....**2** |
| – | Two pairs of dorsal melanophores on each chaetigers. .....**4** |
| 2 | A pair of dorsal melanophores lack ramification; three pairs of black eyes are arranging more or less a straight line..... ***Pseudopolydora paucibranchiata*** |
| – | A pair of dorsal melanophores greatly ramified; lateral and anterior pairs of eyes link each other and form dumbbell-shaped eyes .....**3** |
| 3 | Ramification of dorsal melanophores covering most of dorsal side; a conspicuous large black pigment on pygidium ..... ***Pseudopolydora tsubaki*** |
| – | Ramification of dorsal melanophores not covering most of dorsal side; a conspicuous black pigment spot on pygidium .....***Pseudopolydora* sp.** |
| 4 | A central pair of dorsal black pigment "tilted wheels" shaped in anterior chaetigers; a weak mid-dorsal pigment present from chaetiger VI ..... .....***Pseudopolydora* aff. *achaeta*** |
| – | A central pair of dorsal melanophore dot-like or ramified; mid-dorsal melanophores absent except the first chaetiger.....**5** |
| 5 | Distinct ramified mid-dorsal melanophore present on first chaetiger..... .....***Pseudopolydora* cf. *reticulata*** |
| – | Mid-dorsal melanophore on first chaetiger absent or not distinct and not ramified .....***Pseudopolydora* cf. *kempi*** |

#### *Pseudopolydora* aff. *achaeta* Radashevsky & Hsieh, 2000

Fig. 9A, B

**Larval morphology.** Overall body shape fusiform, head region enlarged due to broad prostomium and expanded lateral lips of vestibule. Prostomium gently notched an-

teriorly. Three pairs of black eyes present in more or less a straight line, most lateral pairs double eyes. Mid-dorsal melanophore on first chaetiger present. Dorsal pigmentation consists of two pairs of lateral and central rows of melanophores. Lateral ones dot-like, beginning on chaetiger II. Central ones shaped like "tilted-wheels" (inverted v-shape) begin on chaetiger III. A central pair of dorsal pigment patches gradually become dot-like on posterior chaetiger. Weak mid-dorsal pigments occur from chaetiger VI. Two medial black pigmentation areas occasionally present ventrally, on approximately chaetiger VI and anterior margin of pygidium. Anterior and posterior margin of prostomium have considerable brown pigment. Black pigment spots occur on sides of prostomium and peristomium. Pygidium has a central black pigment spot. Gastrotrochs on chaetiger III, V, VII, and XII in 13-chaetiger larvae.

**Remarks.** Adult individuals of this species were collected from muddy bottom sediments at 22 m depth in Onagawa Bay in December 2010 and September and December 2011 by using a Smith-McIntyre or Ekman-Birge grab sampler. Adult morphology agrees with the descriptions of *P. achaeta* by Radashevsky and Hsieh (2000) and Abe et al. (2016). However, the 16S rRNA gene sequence obtained in the present study showed a 11.5% (35/304 bp) difference with that of *P. achaeta* from Taiwan (country of type locality), which indicate that these two are different species. Therefore, this species is referred to *P.* aff. *achaeta*. The larvae and adults were confirmed to 100% match using molecular data (Fig. 2).

Planktonic larvae of this species with more than 3-chaetiger stages were abundant in Onagawa Bay during July to November (Abe et al. 2014). A dorsal pigmentation area shaped like "tilted wheels" is a unique characteristic of this species among the known *Pseudopolydora* larvae.

### *Pseudopolydora* cf. *kempi* (Southern, 1921)

Fig. 9C

**Larval morphology.** Overall body shape fusiform, head region enlarged due to broad prostomium and expanded lateral lips of vestibule. Prostomium gently notched anteriorly. Three pairs of black eyes present in more or less a straight line, most lateral pairs double-eyes. Mid-dorsal melanophore on first chaetiger usually absent (Fig. 9C), small non-ramified melanophore present in some individuals. Dorsal pigment consists of four rows of lateral and central pairs of pigment spots. Lateral and central pigments usually begin from chaetigers II and III, respectively. There pigment spots undergo expansion and contraction. Ventral pigment begins on chaetiger III, consists of paired bars on posterior border of each chaetiger. Anterior and posterior margin of prostomium have considerable brown pigment. Black pigment spots occur on sides of peristomium. Pygidium has black central spot. Gastrotrochs on chaetigers V and VII in 13-chaetiger larvae.

**Remarks.** Adult individuals of this species were collected from muddy sediment in Gamo Lagoon in January, May, and December 2011, and April 2013. Adult morphology agrees with the description of *P.* cf. *kempi* by Abe et al. (2016). Therefore, these

individuals were referred to *P.* cf. *kempi*. The 16S rRNA gene sequence obtained in the present study showed a 99.7% (305/306 bp) similarity with that of *P. kempi japonica* Imajima & Hartman, 1964 from Russia (MG460897) reported by Radashevsky et al. (2020b), indicating these two are same species. It will need to be clarified whether *P. kempi* (type locality India) and subspecies *P. kempi japonica* (type locality Japan) are the same species. The larvae and adults were confirmed to 100% match using molecular data (Fig. 2).

Planktonic larvae of this species larger than 12-chaetiger stages were collected from Gamo Lagoon in August 2012. The larval morphology of this species observed in the present study agrees with the descriptions of *P. kempi* by Blake and Woodwick (1975) and of *P.* cf. *kempi* by Kondoh et al. (2017). These species have adelphophagic and lecithotrophic larval development, in which larvae feed on nurse eggs in brood capsules, hatch at a very late stage, and have a short pelagic life (Blake and Woodwick 1975; Kondoh et al. 2017). Reproduction and larval development of these species under the name of *P. kempi* and *P. kempi japonica* were also described by Srikrishnadhas and Ramamoorthi (1977), Myohara (1979), and Radashevsky (1985). However, the larvae of species in these descriptions resemble those of *Pseudopolydora* cf. *reticulata* Radashevsky & Hsieh, 2000 described by Kondoh et al. (2017) and of the present study in having planktotrophic development without nurse eggs and distinct dorsal melanophores including a middorsal melanophore on the first chaetiger. The taxonomy of *P. kempi* is unclear because its original description is quite brief, and the current location of type specimen is unknown (Radashevsky and Hsieh 2000). Therefore, studies resolving the taxonomy of *P. kempi* are necessary.

### *Pseudopolydora paucibranchiata* (Okuda, 1937)

Fig. 9D

**Larval morphology.** Overall body shape fusiform, head region enlarged due to broad prostomium and expanded lateral lips of vestibule. Prostomium gently notched anteriorly. Three pairs of black eyes present in more or less a straight line, most lateral pair comma-shaped. A mid-dorsal ramified melanophore on chaetiger I. A pair of melanophores present dorso-laterally from chaetigers II onwards. Black pigment spots occur on lateral surface of chaetiger II, on sides of peristomium, and pygidium. Two small medial black pigment spots occasionally present ventrally on approximately chaetiger VI and anterior margin of pygidium. Gut has yellow-green color due to ingested food. Gastrotrochs on chaetiger V, VII, and XI in 13-chaetiger larvae.

**Remarks.** Adult individuals were collected from muddy bottom sediment in the intertidal zone of Mangoku-ura Inlet in July 2014. Adult morphology agrees with the description of *Pseudopolydora paucibranchiata* by Okuda (1937, as *Polydora*). Therefore, these individuals were referred to this species. The larvae and adults were confirmed matching (18S: 1784/1784, 16S: 454/455 bp) using molecular data (Fig. 2).

The planktonic larvae of this species were reported to be common in Onagawa Bay during June to November (Abe et al. 2014). The larval morphology of this species observed in the present study agrees with the descriptions by Blake and Woodwick (1975), Ward (1977), Myohara (1980), Wu and Chen (1980), Radashevsky (1983), and Blake (2006). The dorsal pigment pattern of this species consists of one pair of melanophores, which agrees with that of the larvae of *Pseudopolydora vexillosa* Radashevsky & Hsieh, 2000 photographed by Mok et al. (2009) and Chandramouli et al. (2011, 2013), currently synonymized to *P. paucibranchiata* (Junqueira et al. 2009). The dorsal pigment pattern of these larvae is also similar to that of the larvae of *Pseudopolydora antennata* (Claparède, 1869) described by Hannerz (1956, as *Polydora antennata*), but the latter species has a more thickened body shape compared to the former.

### *Pseudopolydora* cf. *reticulata* Radashevsky & Hsieh, 2000

Fig. 9E, F

**Larval morphology.** Overall larval shape fusiform, head region enlarged due to broad prostomium and expanded lateral lips of vestibule. Prostomium slightly notched anteriorly. Three pairs of black eyes present in more or less a straight line, most lateral pairs double-eyes. Large patches of black pigment on lateral peristomium present. Middorsal melanophore on chaetiger I usually present. Dorsal pigments undergo expansion and contraction, expanding to cover almost complete dorsal surface with finely ramified black pigment (Fig. 9F) or contract to dot-like black pigmentation without ramifications (Fig. 9E). Ventral pigment usually absent, consisting of paired bars on the posterior border on anterior chaetigers occasionally present. Black pigment on pygidium. Gastrotrochs on chaetigers V, VII, and XII in 17- and 18-chaetiger larvae, late larvae lose gastrotrochs on chaetigers V and/or XXII.

**Remarks.** Adult individuals of this species were collected from muddy sediment in Gamo Lagoon in April 2013 and Sasuhama in July and September in 2011. Adult morphology agrees with the description of *P.* cf. *reticulata* by Abe et al. (2016). Therefore, these individuals were referred to this species. The 16S rRNA gene sequence obtained in the present study showed a 99.4% (304/306 bp) similarity with that of *P. bassarginensis* (Zachs, 1933) from Russia (MG460894) reported by Radashevsky et al. (2020b), indicating these two are one species. Although the Japanese population shows intermediate morphological characteristics between *P. reticulata* (type locality Taiwan) and *P. bassarginensis* (type locality Russia), Abe et al. (2016) tentatively identified the Japanese population as *P.* cf. *reticulata* because the original description of *P. bassarginensis* is very brief and the status of the species remains unclear. The results of the present study indicate that the Japanese population likely belongs to *P. bassarginensis*, but whether the morphologically similar *P. reticulata* and *P. bassarginensis* are considered molecularly as the same or different species will need to be clarified. Planktonic larvae of *P.* cf. *reticulata* larger than the 3-chaetiger stage were collected from Gamo

Lagoon, Sasuhama, and Onagawa Bay mainly from July to September. The larvae and adults were confirmed to match (18S: 1775/1775, 16S: 468/470 bp) using molecular data (Fig. 2).

*Pseudopolydora* cf. *reticulata* and *P.* cf. *kempi* are very similar sister species; specimens from Japan once misidentified as *P.* cf. *kempi* or *P. kempi japonica* were distinguished based on their morphology and 18S and 28S rRNA gene sequences by Abe et al. (2016). The larvae of these two species are also quite similar, but the mid-dorsal melanophore on chaetiger I is usually present in *Ps.* cf. *reticulata* and absent in *P.* cf. *kempi*; moreover, the dorsal pigmentation is more distinct in the former species than in the latter. The two species also differ in reproduction and larval development: *P.* cf. *kempi* has lecithotrophic development with a short planktonic phase, whereas *P.* cf. *reticulata* has planktotrophic development with a long planktonic phase (Kondoh et al. 2017).

### *Pseudopolydora tsubaki* Simon, Sato-Okoshi & Abe, 2017

Fig. 9G, H

**Larval morphology.** Overall larval shape fusiform, head region enlarged due to broad prostomium and expanded lateral lips of vestibule. Prostomium gently notched anteriorly. Three pairs of black eyes present, comprising one pair of rounded median eyes, one pair of large lateral eyes, and one pair of large anterior eyes. Lateral and anterior pairs of eyes link with each other and form dumbbell-shapes almost divided into two equal parts by a deep constriction. Mid-dorsal ramified melanophore present on chaetiger I in early larvae. Mid-dorsal melanophore on chaetiger I occasionally absent or expanded to finely ramified melanophore in late larvae. A paired of melanophores occur dorso-laterally from chaetiger II onwards, usually finely ramified in late larvae (Fig. 9H). Ramified melanophores cover almost entire ventral surface on chaetigers III–VII in 11-chaetiger larvae. Black pigment spots on sides of peristomium absent. Conspicuous large black pigment on pygidium. Gastrotrochs on chaetigers V and VII in 11-chaetiger larvae.

**Remarks.** Adult individuals were collected from mud deposits in crevices of shells of living *C. gigas* oysters in Habu Port, Izu-Oshima Island, and Tomiura, Boso Peninsula in April 2016. Adult morphology agrees with the description of *Pseudopolydora tsubaki* by Simon et al. (2019a). Therefore, these individuals were identified as *P. tsubaki*. The larvae and adults were confirmed to 100% match using molecular data (Fig. 2).

A small number of planktonic larvae of this species were collected in Habu Port and Tomiura in May and June 2016. The larvae of *P. tsubaki* are similar to those of *Pseudopolydora pulchra* (Carazzi, 1893) in having ramified melanophores covering the ventral side; however, these cover only the central part of the body in the former species, whereas those of latter species cover the ventral surface almost entirely (Hannerz 1956, as *Polydora pulchra*; Rullier 1963, as *Polydora pulchra*). The dorsal pigment pattern is also different in these two species: two pairs of melanophores are distinct in *P. pulchra*, whereas the melanophore pair is ambiguous in *P. tsubaki*.

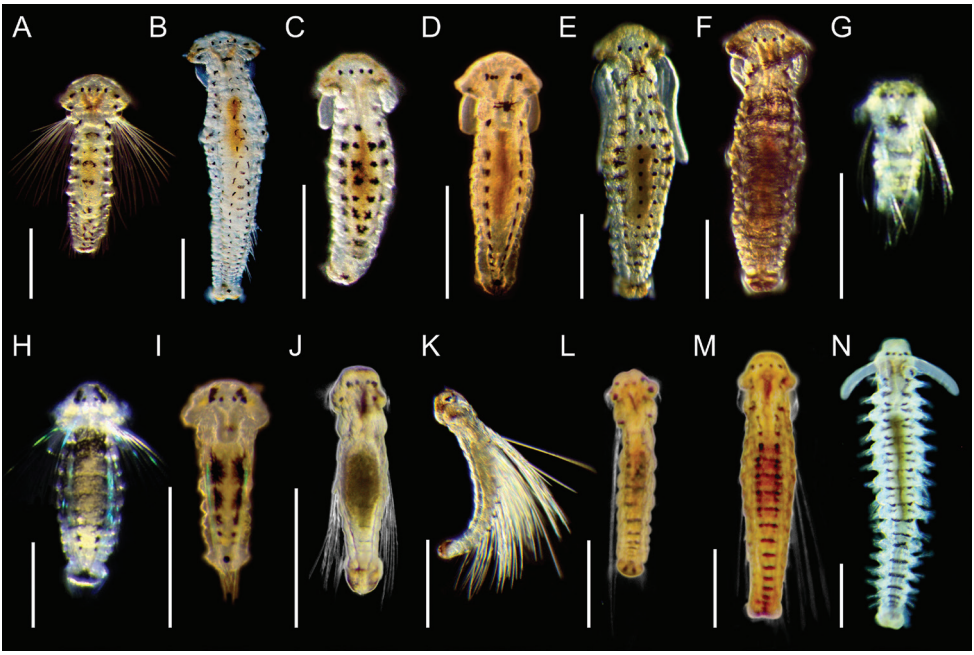


**Figure 9.** Light micrographs showing the morphologies of living spionid larvae of genera *Pseudopolydora* and *Spio* **A, B** *Pseudopolydora* aff. *achaeta*, dorsal view of 12-chaetiger (**A**) and 25-chetiger larvae (**B**) **C** *Pseudopolydora* cf. *kempi*, dorsolateral view of 12-chetiger larva **D** *Pseudopolydora paucibranchiata*, dorsal view of 13-chaetiger larva **E, F** *Pseudopolydora* cf. *reticulata*, dorsal view of 17-chaetiger (**E**) and 16-chaetiger larvae (**F**) **G, H** *Pseudopolydora tsubaki*, dorsal view of 5-chaetiger (**G**) and 11-chaetiger larvae (**H**) **I** *Pseudopolydora* sp., dorsal view of 7-chaetiger larva **J, K** *Spio* sp. 1, dorsal view of 8-chaetiger (**J**) and lateral view of 12-chaetiger larvae (**K**) **L–N** *Spio* sp. 2, dorsal view of 10-chaetiger (**L**) and 17-chaetiger larvae (**M**), and 17-chaetiger metamorphosing larvae (**N**). Scale bars: 300 μm.

### *Pseudopolydora* sp.

Fig. 9I

**Larval morphology.** Overall larval shape slightly fusiform, head region enlarged due to broad prostomium and expanded lateral lips of vestibule. Prostomium rounded anteriorly. Three pairs of black eyes present, comprising one pair of rounded median eyes, one pair of large lateral eyes, and one pair of large anterior eyes. Lateral and anterior pairs of eyes link with each other and form a dumbbell-shape almost divided into two equal parts by a deep constriction. Small mid-dorsal melanophore present on chaetiger I. A distinct paired melanophore occurs dorso-laterally from chaetiger II onwards, ramified in anterior chaetigers. Black pigment spots on sides of peristomium absent. Dot-like black pigmentation on pygidium.

**Remarks.** No benthic adult stages were collected in the present study. The larvae formed a monophyletic clade with the other *Pseudopolydora* species with > 50% bootstrap support (Figs 2, 3). Therefore, this species was identified as a member of *Pseudopol-*

*ydora*. As the 18S rRNA gene sequences obtained from the larvae did not match any of the available *Pseudopolydora* sequences, this species is referred to *Pseudopolydora* sp.

Only one larva individual was collected from Sasuhama in August 2011. The dorsal pigment pattern of this larva somewhat resembles that of *P. paucibranchiata*; however, the mid-dorsal pigment of this species is weaker and its dorsolateral melanophores are more ramified than those of *P. paucibranchiata*. The eye arrangement of this larva resembles that of late *P. tsubaki* larvae: three pairs of black eyes are present, but not in a straight line.

## Genus *Spio* Fabricius, 1785

**Larval diagnosis.** Overall body shape long, slender, and weakly or moderately fusiform. Prostomium small and rounded anteriorly. Lateral part of peristomium weakly demarcated from prostomium. Three pairs of black eyes present, most lateral often double-eyes. Dorsal pigmentation consists of transverse band-shaped or dot-like paired lateral melanophores. Some species lack black pigmentation. Ventral pigment usually absent. Dark-brown pigment may be present on pygidium. Nototrochs occur in all chaetigers except first one or two chaetigers, where nuchal organs develop. Gastrotrochs occur regularly in every other chaetiger from chaetiger III onwards. Larval chaetae on first chaetiger usually fairly long. Branchiae develop in late larvae, first on chaetiger II or III. One pair of anal cirri present on pygidium in late larvae (Thorson 1946, as spionid larva C, E, and F; Hannerz 1956; Wu et al. 1965; Simon 1963, 1967, 1968; Guérin 1972; Srikrishnadhas and Ramamoorthi 1981; Plate and Husemann 1994).

### Identification key to species of the larvae belonging to the genus *Spio* in northeastern Japan

1 Two rows of black melanophore spots on each side of dorsum from chaetiger I onwards, linking by band-shaped medial black pigmentation from chaetiger IV or V ..... ***Spio* sp. 2**

– Dorsal black melanophores not distinct; rows of faint transverse band-shaped black pigmentation present on dorsum from chaetiger IV onwards .... ***Spio* sp. 1**

### *Spio* sp. 1

Fig. 9J, K

**Larval morphology.** Overall larval shape slender and weakly fusiform. Larval chaetae on first chaetiger fairly long. Prostomium round anteriorly. Small patches of black pigment on peristomium ventrally. Three pairs of black eyes present, most lateral pairs double-eyes. Distinct black melanophore absent, rows of faint transverse band-shaped black pigmentation on dorsum from chaetiger IV onwards. Pharynx exhibits weak dark or brownish pigmentation. Gut yellow-green in color due to ingested food.

**Remarks.** Adult individuals of this species were collected from Rishiri Island, northern Japan, in July and August 2017. These specimens were previously identified as *S. arndti* Meißner, Bick & Bastrop, 2011 (Abe et al. 2019c) since adult morphology agreed. Although 18S rRNA gene sequence obtained in the present study 100% match with that of *S. arndti* (FR823434, 1761/1761 bp), because the 16S rRNA gene sequences were different (6.7%, 30/451 bp), the species reported here is referred to *Spio* sp. 1. The 16S rRNA gene sequence of *Spio* sp. 1 was rather more similar (96.1%, 298/310 bp) to that of *Spio* sp. 2573 from Russia (KT200126), but conspecificity of these two is unclear. A few planktonic larvae of this species were collected from Onagawa Bay only in May 2011. The larvae and adults were confirmed to match (18S: 1762/1762, 16S: 466/467 bp) using molecular data (Fig. 2).

The absence of distinct black melanophores in larvae of this species differentiates them from those of *Spio* sp. 2 (see below). Slight dorsal pigmentation was also reported in adelphophagic benthic larvae of *Spio setosa* Verrill, 1873 sensu Simon (1967, 1968), which were essentially unpigmented, and in those of *Spio multioculata* (Rioja, 1918) described by Hannerz (1956). However, the larval morphologies of these two species are different from that of *Spio* sp. 1 in lacking ventral black pigment on the peristomium (in both former species) and long larval chaetae on the first chaetiger (in *S. setosa*), and in having a relatively thickened body shape (in both species).

### *Spio* sp. 2

Fig. 9L–N

**Larval morphology.** Overall larval shape elongated, slender, weakly fusiform. Larval chaetae on first chaetiger fairly long. Prostomium round anteriorly. Small patches of black pigment on lateral peristomium present ventrally. Three pairs of black eyes present, most lateral pairs double-eyes. Two rows of dot-like black melanophores on each side of dorsum from chaetiger I onwards, linking by band-shaped medial black pigmentation from chaetiger IV or V. Pharynx exhibits weak dark or brownish pigmentation. The larvae which are ready to metamorphose have branchiae from chaetiger II, pigment spot on palps, and a pygidium with four leaf-shaped anal cirri.

**Remarks.** Adult individuals were collected from muddy sand sediments of shallow water in Sasuhama in September 2011. These adults were morphologically identified as a *Spio* species, but they could not be identified to species level. *Spio* spp. 1 and 2 are distinguishable morphologically by the number of ventral epidermal glands. The 18S and 16S rRNA gene sequences obtained in the present study did not match any of the available *Spio* sequences (Figs 2, 3). The larvae and adults were confirmed to 100% match using molecular data (Fig. 2).

Planktonic larvae of this species were found in Sasuhama and Onagawa Bay from April to August during the study period. Larval morphology and pigmentation pattern of this species is similar to that of *Spio decorata* Bobretzky, 1870 described by Guérin 1972. However, the latter species was originally described from the Black Sea and has not been recorded in Japan.

## Discussion

### Larval identification based on the molecular data

The present study identified 41 species from 14 genera of planktonic spionid larvae by comparing adult and larval gene sequences and revealed high diversity of spionid larvae in neritic plankton communities (Table 1, Figs 2, 3). Planktonic spionid larvae of several species could not be identified to species level because of the lack of adult reference sequences or difficulties in adult identification. As the genetic information available for many marine invertebrate taxa including polychaetes is insufficient, the increase in gene sequence data based on accurate species identification and the establishment of a comprehensive database of adult reference sequences are essential for a more precise and efficient larval molecular identification. However, most of the larvae from the present study that did not have sequences that matched those of adults were identified to genus level based on their position within the phylogenetic tree; this was only possible because many of the spionid genera were recovered well or moderately supported monophyletic groups in our molecular phylogenetic analyses (Figs 2, 3). In contrast, the monophyly of some spionid taxa, particularly of the genera *Dipolydora*, *Malacoceros*, and *Prionospio* were ambiguous and not well supported in the phylogenetic tree recovered herein. It should be noted that *Malacoceros indicus* and *Malacoceros* cf. *indicus* were recovered as quite distant from *Malacoceros fuliginosus* and *Malacoceros* sp. (Fig. 3), potentially indicating the paraphyletic origins of these two clades. The results of the phylogenetic analyses also showed that the monophyly of subfamily Nerininae is doubtful and more likely to be paraphyletic. Because intergeneric phylogenetic relationships were ambiguous due to the low statistical support of most of the higher internal nodes (Figs 2, 3), it was difficult to compare with the previous results of phylogenetic relationships among spionid genera provided by Sigvaldadóttir et al. (1997) and Blake and Arnofsky (1999). The results of our phylogenetic analyses reinforce the need for a more robust and comprehensive molecular phylogenetic study of this taxon to test the monophyly of each genus and subfamily and to shed light on the phylogenetic relationships among spionid genera.

In the present study, many spionid species were collected as planktonic larval stages. This emphasizes the effectiveness of field investigations of both larval and adult stages to assess the cryptic species diversity in benthic invertebrate fauna of coastal waters. The reference gene sequences used in the present study for adults covered most of the species belonging to the genera *Polydora* and *Pseudopolydora* hitherto recorded from Japan (Sato-Okoshi 1999, 2000; Sato-Okoshi and Abe 2012, 2013; Teramoto et al. 2013; Abe et al. 2016; Simon et al. 2019a). However, the sequences of some *Polydora* and *Pseudopolydora* larvae, namely *Polydora* sp. 2, *Polydora* sp. 3, and *Pseudopolydora* sp., did not match any adult reference sequences. This emphasizes the need for detailed taxonomic studies with a more comprehensive sampling of spionid adults to reveal the actual biological diversity of this taxon in Japan.

## Morphology of spionid larvae

The family Spionidae can be divided into two subfamilies: 1) Spioninae Söderström, 1920, which includes the genera *Spio*, *Microspio* Mesnil, 1896, *Pygospio* Claparède, 1863, and genera of the tribe Polydorini; and 2) Nerininae Söderström, 1920, which includes almost all remaining spionid genera, except for *Atherospio* Mackie & Duff, 1986 and *Pygospiopsis* Blake, 1983 (Blake 2006), besides *Poecilochaetus* and *Trochochaeta*, which were recently placed within the family Spionidae (Radashevsky et al. 2018). The larvae of these two subfamilies were distinguished in the present study based on color and number of eyes, body pigmentation, shape of peristomium, and distribution of gastrotrochs, and by the following characteristics identified by Hannerz (1956): larvae of Spioninae have three pairs of black eyes (lateral eyes are often double eyes), distinct black pigmentation with melanophores, lateral parts of the peristomium not demarcated from prostomium, and gastrotrochs present from chaetiger III, V, or VII onwards, but absent in all of the succeeding chaetigers (Figs 7–9); larvae of Nerininae have two pairs of red or dark red eyes, lack distinct black pigmentation, lateral parts of the peristomium are well developed and often demarcated from prostomium, and gastrotrochs present from chaetiger II or III onwards and in all succeeding chaetigers (Figs 4, 5). Blake (1969) also discussed the presence of ventral ciliary patches in early larval stages as a common characteristic of subfamily Spioninae, but these cilia were not herein observed because they are lost in early larval stages.

Hannerz (1956) reported the following exceptions to the abovementioned typical larval morphologies: larvae of *Prionospio fallax* Söderström, 1920 (as *P. malmgreni*, see Blake and Arnofsky 1999) with two pairs of black eyes; larvae of *Malacoceros* (as *Scolelepis*), which belongs to Nerininae, with intermediate characteristics between the two subfamilies, i.e., with three pairs of black eyes and gastrotrochs regularly distributed on every other chaetiger as in Spioninae larvae. However, Plate and Husemann (1994) reported that the larvae of *Malacoceros fuliginosus* (Claparède, 1868) have up to three pairs of red eyes in early stages and that eye color changes to black as larvae develop. Radashevsky and Migotto (2006) reported that the larvae of *Malacoceros* sp. have two pairs of red eyes. In the present study, the larvae of *Rhynchospio* have two pairs of dark red eyes (Fig. 4F, G), whereas their morphology resembled those of *Malacoceros* species described by Hannerz (1956). Radashevsky (2007) also reported that larvae of *Rhynchospio nhatrangi* have two pairs of red eyes. Besides the various reports on the number and color of eyes, the close relationships of *Malacoceros* and *Rhynchospio* to the subfamily Spioninae were indicated by larval morphology, and results of the phylogenetic analyses presented (Figs 2, 3) also provide some support for this hypothesis.

In the subfamily Spioninae, the most obvious larval differences between genera and species are the overall body shape and type and arrangement of pigmentation (Blake and Arnofsky 1999). The overall body shape of larvae of *Polydora*, *Dipolydora*, and *Spio* tended to be long and slender, whereas those of *Boccardiella*, *Boccardia*, and *Pseudopolydora* tended to be thick and fusiform (Figs 7–9), although

*Boccardia proboscidea* (Fig. 7A, B) and *Boccardia* sp. 1 (Fig. 7D–F) showed relatively slender body shapes. The lateral enlargement of the prostomium in Spioninae is variable: large in *Boccardiella* and *Pseudopolydora*, moderate in *Polydora* and *Boccardia*, and small in *Dipolydora* and *Spio*. Fairly long larval chaetae on the first chaetiger are highly characteristic of *Spio* within Spioninae.

The dorsal black pigmentation with melanophores is distinct in the subfamily Spioninae, and the pattern of rows of melanophores is generally diagnostic among Spioninae genera. The typical patterns of dorsal pigmentation rows in larvae are as follows: a pair of transverse bands of black pigment on some anterior chaetigers followed by a pair of large branching melanophores in *Polydora*; lack of large melanophores, but with a pair of medial spots or bands, a pair of lateral pigment patches, and mid-dorsal black pigment spot continuing posteriorly from the anterior chaetigers in *Dipolydora*; mid-dorsal melanophores arranged in a single row in *Boccardia*; medial and lateral pairs of spots or bands with black pigmentation and a small patch of pigment at the base of the notopodia present on almost all chaetigers in *Boccardiella*; a mid-dorsal melanophore on the first chaetiger, and one or two pairs of melanophores on each chaetiger in *Pseudopolydora*; a pair of black pigment spots and transverse black pigment bands linking them on each chaetiger in *Spio* (Figs 7–9). These typical dorsal pigment patterns were also reported in many previous studies (Hannerz 1956; Blake 1969, 2006; Blake and Arnofsky 1999; and references cited therein). However, unusual larval pigment patterns are often found in members of each of the aforementioned genera; therefore, these typical larval pigment patterns are not wholly consistent within each genus. For example, the single row of dorsal melanophores typical of *Boccardia* larvae was also observed in larvae of *Polydora* cf. *glycymerica* (Fig. 8E) and *Dipolydora* cf. *commensalis* (Fig. 7L), and have been reported in *Polydora glycymerica* (Radashevsky 1989), *Polydora hermaphroditica* (Hannerz 1956; Plate and Husemann 1994), *Dipolydora commensalis* (as *Polydora commensalis*: Andrews 1891; Hatfield 1965; Blake 1969; Radashevsky 1989), and *Pseudopolydora rosebelae* Radashevsky & Migotto, 2009. In contrast, the single row of dorsal melanophores is absent in *Boccardia chilensis* Blake & Woodwick, 1971 (Carrasco 1976; Blake and Kudenov 1981), *Boccardia pseudonatrix* (Fig. 7C), and *Boccardia semibranchiata* Guérin, 1990 (Guérin 1991). The larvae of *Pseudopolydora* cf. *kempi* lack a mid-dorsal melanophore on the first chaetiger, which is typical in *Pseudopolydora* larvae (Fig. 9C; Kondoh et al. 2017). The distinct dorsal black pigment is absent in *Spio setosa* Verrill, 1873 (Simon 1967, 1968) and *Spio* sp.1 (Fig. 9J, K).

Larvae of the following Spioninae genera were not collected in the present study: *Microspio*, *Pygospio*, and the polydorid genera *Amphipolydora* Blake, 1983, *Carazziella* Blake & Kudenov, 1978, *Polydorella* Augener, 1914, and *Tripolydora* Woodwick, 1964 (among them, *Microspio* and *Carazziella* have records from Japan by Okuda 1937, Sato-Okoshi 1998). Little is known about the larval morphology of the genera *Amphipolydora*, *Polydorella*, and *Tripolydora*. The larvae of *Microspio* resemble those of *Spio* in having a long and slender body shape and band-shaped dorsal black pigmentation (e.g., Hannerz 1956; Cazaux 1971). The larvae of *Carazziella* resemble those of the polydorid genus *Boccardia* in having a fusiform body shape and a single row of dorsal

melanophores (Carrasco 1976, as *Polydora citrona*; Blake and Arnofsky 1999; Blake 2006). The morphology of planktonic larval stages of *Pygospio elegans* as described in Hannerz (1956) resembles that of *Pseudopolydora* in having a thick and fusiform body shape, laterally enlarged prostomium, and mid-dorsal melanophore on the first chaetiger. Blake (1969) also noted the morphological similarity between the larvae of *Pseudopolydora* and *Pygospio elegans* and suggested the possibility that polydorids are closely related to *Pygospio* through *Pseudopolydora*. Subsequently, Blake and Woodwick (1975) reported the similarities of nurse egg feeding patterns between *Pseudopolydora kempi* and *Pygospio elegans*, further strengthening the view of a close relationship between these two genera. This hypothesis is supported by the results of the phylogenetic analysis presented, showing that polydorids plus *Pygospio* form a monophyletic clade with robust statistical supports (Fig. 3).

In the subfamily Nerininae, as in Spioninae, the most obvious differences among genera are also regarding their overall body shapes. The lateral parts of the peristomium are conspicuous, well developed, and distinctly demarcated from the prostomium in larvae of *Laonice*, *Rhynchospio*, and *Scolelepis*, but they are less pronounced in those of *Aonides*, *Paraprionospio*, *Prionospio*, and *Spiophanes*, as previously noted by Hannerz (1956). Larvae of the former group of genera (*Laonice*, *Rhynchospio*, *Scolelepis*) also have a relatively wide body shape, whereas those of the latter group have a narrow body shape. Regarding the larvae of the former group, the prostomium is more or less stumpy and not pointed anteriorly in *Rhynchospio* and *Laonice*; however, *Scolelepis* larvae have a unique body shape distinct from other spionid genera and their prostomium is pointed anteriorly, terminating in a tapered tip, and the lateral parts of the peristomium are demarcated and bearing a large peristomial umbrella. The larvae from the latter group (*Aonides*, *Paraprionospio*, *Prionospio*, *Spiophanes*), *Paraprionospio*, and *Prionospio* characteristically have extremely long and thin bodies with numerous chaetigers. In particular, larvae of *Paraprionospio* are extremely large in terms of body size and chaetiger number at metamorphosis among the spionid larvae (Yokoyama 1981). The larval morphology of the genus *Poecilochaetus* resembles that of *Paraprionospio* and *Prionospio*: larvae have extremely long and slender transparent bodies without distinct black pigmentation. However, the first differs from the other two in having small lateral pigment spots on each side of the chaetigers, a long metatrochophore stage (up to ca. 30–40 chaetiger stages), and a serpentine swimming behavior with developed parapodia bearing cirriform dorsal and ventral postchaetal lobes in the nectosoma stage. *Poecilochaetus* larvae are distinctive among spionid larvae in having gastrotrochs from chaetiger I onwards despite all other Nerininae larvae having gastrotrochs from chaetiger II or III onwards. In the present study, a pair of lateral processes on the prostomium developed in late larvae was found only in larvae of *Rhynchospio* and *Spiophanes*, it also previously described for *Malacoceros* larvae (Hannerz 1956). Fairly long and straight larval chaetae on the first chaetiger are highly characteristic of *Aonides* larvae, especially in the early stages (Fig. 4A, B); however, similar long and straight larval chaetae were also herein observed in larvae of *Spio* sp. 2 (Fig. 9L, M). Although larvae of the genera *Malacoceros*, *Marenzelleria* Mesnil, 1896, *Streblospio* Webster, 1879, and *Trochochaeta* (subfamily Nerininae) were not collected in the present study, larval morphologies of

these genera have been well described in previous studies. The larvae of *Malacoceros* resemble those of *Rhynchospio* (see above). The larvae of *Marenzelleria* (Bochert and Bick 1995) resemble those of *Laonice* in having the following characters: the remains of the egg membrane visible in early stages; in late larvae, lateral parts of the peristomium are conspicuous, well developed and distinctly demarcated from prostomium, and palps start developing laterally on the peristomium; the body is broader than the prototroch; both notopodial and neuropodial larval chaetae are present. However, the larvae of these two genera differ in the arrangement of nototrochs and gastrotrochs. The larval morphology of the genus *Streblospio*, which is included in the *Prionospio* complex (Dean 1965; Blake and Arnofsky 1999; Blake 2006) resembles that of *Prionospio*. The larval morphology of the genus *Trochochaeta* is distinctive among spionid larvae in having unusually long larval chaetae on the first chaetiger, very pronounced peristomial umbrella with two rows of robustly developed prototrochs, and total absence of nototrochs; larvae of this genus also present the typical morphological characteristics of Nerininae larvae, such as two pairs of red eyes, lack of distinct black pigmentation, and gastrotrochs from chaetiger II onwards on all succeeding chaetigers, although gastrotrochs of *Trochochaeta* larvae are weakly developed and those on chaetiger II are especially small and inconspicuous in late-stage larvae (Hannerz 1956, as *Disoma*; Blake and Arnofsky 1999; Blake 2006).

There is insufficient information on the larval morphology of the remaining genera of Nerininae. The larval development and morphology of *Dispio uncinata* Hartman, 1951 were described, and this species' close relationship with *Aonides* was suggested by Blake and Arnofsky (1999) and Blake (2006). However, Radashevsky et al. (2011) pointed out that the larvae described by these authors are most likely those of *Aonides californiensis* Rioja, 1947 rather than of a *Dispio* Hartman, 1951 species. Species-level identification of larvae from this subfamily is generally more difficult because of the lack of structured pigmentation, which is a useful characteristic for identifying species of Spioninae larvae. Especially in the genus *Prionospio*, larval morphology is quite simple and similar among species, which made it impossible to find morphological characters to distinguish between them in the present study.

Except for *Prionospio* spp., most of the planktonic spionid larvae collected in the present study have morphological characteristics that could be used to distinguish genera and species, and allowed morphological identification based on overall body shape and pigment patterns. The present paper provides identification keys to genera and species of planktonic spionid larvae from northeastern Japan; however, sufficient attention to developmental and/or intraspecific variation of larval morphological characteristics and the disappearance of pigments after fixation (only the black pigment usually remains after fixation) is required for accurate larval identification.

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# Description of *Hemienchytraeus wuhanensis* sp. nov. (Annelida, Clitellata, Enchytraeidae) from central China, with comments on species records of *Hemienchytraeus* from China

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## Abstract

*Hemienchytraeus wuhanensis* **sp. nov.** is described from hardwood forest soil in Wuhan, China. This moderately sized enchytraeid species of 6–9 mm body length is characterized by: (1) an oesophageal appendage with tertiary branches, (2) three pairs of secondary pharyngeal gland lobes in V, VI, VII, (3) five pairs preclitellar nephridia, from 5/6 to 9/10, (4) dorsal vessel originating in clitellar segments, (5) a girdle-shaped clitellum, (6) a relatively small male reproductive apparatus without seminal vesicle, and (7) spermathecae that extend to VI–VII. DNA barcodes of paratype specimens of the new species are provided. Previous species records of *Hemienchytraeus* from China are critically discussed.

## Keywords

DNA barcoding, Oligochaeta, new species, taxonomy

## Introduction

*Hemienchytraeus* Černosvitov, 1934 is a well-defined genus mainly distributed in the tropical and subtropical regions (Healy 1996; Xie et al. 1999; Schmelz and Römbke 2005). In Enchytraeidae it belongs, according to a molecular phylogenetic analysis (Erséus et al. 2010), to a clade separate from most other genera, but together with *Achaeta*. The genus is distinguished by the following characters: (1) head pore on prostomium; (2) two chaetae per bundle; (3) oesophageal appendage unpaired in III dorsally, behind pharyngeal pad, bifurcating into two primary branches, each of them usually branching into two or more secondary branches, and these sometimes with tertiary branches; (4) nephridial anteseptale large, with coils of canal; (5) no intestinal diverticula; (6) spermathecae free, blind-ending, ampulla without diverticula; (7) sperm funnel usually tapering distad (Schmelz and Römbke 2005; Schmelz and Collado 2010).

To date, 24 species have been reported worldwide (Schmelz and Römbke 2005; Dózsa-Farkas and Hong 2010; Schmelz et al. 2015). These species are mainly distributed in America (12 species), Asia (11 species), Africa (3 species), and Europe (2 species). Six species have been reported from China so far: *H. stephensoni* Cognetti, 1927, *H. bifurcatus* Nielsen & Christensen, 1959, *H. loksai* Dózsa-Farkas, 1989, *H. theae* Prabhoo, 1960, *H. planisetosus* Xie et al., 1999, and *H. brachythecus* Xie et al., 1999 (Wang and Cui 2007). Of these, the latter two are only known from China. In this paper, we add a new member to this list, which was collected from Wuhan, China. We describe the morphology of the species and compare it with congeners. We also provide COI sequences of *Hemienchytraeus wuhanensis* sp. nov. and calculate genetic distances using the sequences of *Hemienchytraeus* spp. available in GenBank. Finally, we comment on species finds of *Hemienchytraeus* spp. in China.

## Materials and methods

Soil samples were collected at forest sites at the Huazhong Agricultural University and Wuhan University, Wuhan, in April 2019. The samples were directly scooped using a steel shovel to a depth of ca 15 cm, placed in a breathable cloth bag and taken to the laboratory and stored at 4 °C. Worms were extracted from soil using a standard hot wet funnel extracting device (O'Connor 1962; Healy and Rota 1992). All worms were examined and identified alive. Body size, colour, movement, and maturity were observed with a Zeiss Stemi 508 stereomicroscope. Other characters were examined, measured, and photographed with a Zeiss Axio Imager A2 microscope using differential interference contrast optics and a Zeiss Axiocam 305 color digital camera with ZEN 2011 Blue Version software. The specimens were then anaesthetized in 30% ethanol and preserved in 75% ethanol (Dózsa-Farkas and Hong 2010). For taxonomic observation, some mature specimens were stained with borax-carmine, dehydrated in an ethanol series from 70% to absolute, mounted temporarily in clove oil and permanently mounted in neutral balsam (Dózsa-Farkas et al. 2015; Zhang et al. 2018).

Drawings from whole mounts were made with the help of an Olympus drawing tube. Type material is deposited in the Museum of Aquatic Organisms (MAO), Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China.

Total genomic DNA was extracted from five entire individuals respectively, using TIANamp Micro DNA Kit (Tiangen Biotech, Beijing, China). The COI gene was amplified from each DNA extract with primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994). These five specimens, of which no morphological parts are left, are part of the type series, as paratypes. Eight COI gene sequences of four different species in genus *Hemienchytraeus* were downloaded from GenBank, alignments were trimmed (resulting alignments were 591bp), aligned and K2P genetic distances were calculated using MEGA-X (Kumar et al. 2018).

Unless specified otherwise, measurements refer to mature fixed specimens (both whole-mounts and dissected specimens). When "*in vivo*" is given, measurements refer to living specimens.

## Taxonomy

***Hemienchytraeus wuhanensis* sp. nov.**
http://zoobank.org/D3137BCA-E1CC-4FC7-AA55-A88FA9ED06E6

**Holotype.** Fully mature, whole-mounted specimen, stained, HBO201904002.

**Type locality.** Mount Shizi, litter layer of hardwood forest (30°28'42.57"N, 114°21'10.48"E; 44 m a.s.l.), Huazhong Agricultural University (Fig. 1), Wuhan, Hubei Province, 6 April 2019, coll. Y. H. Ge.

**Paratypes.** HBO201904003, HBO201904004 two whole-mounted fully mature specimens, HBO201904005–HBO201904007, three adult specimens, used entirely for DNA extraction; HBO201904008–HBO201904010 three adult specimens from the type locality maintained in 75% alcohol, same data as holotype. HBO201904001 one whole mounted fully mature specimen, HBO201904011–HBO201904012 two adult specimens used for extract DNA, and HBO201904013–HBO210904015 three adult specimens maintained in 75% alcohol from Mount Luojia, under a pine tree (30°32'05.39"N, 114°22'10.95"E; 31 m a.s.l.), Wuhan University, Wuhan, Hubei Province, 2 April 2019, coll. X. K. Jiang & J. J. Chen.

**Etymology.** Named after the city where the species was found.

**Distribution and habitat.** Mineral soil and organic layers under camphor trees near a narrow, tarred road at Mount Shizi, Huazhong Agricultural University; mineral soil and organic layers under pine trees at Mount Luojia, Wuhan University. The two hills are about 10 km apart, with little human disturbance.

**Diagnosis.** This new species can be recognized by the following combination of diagnostic traits: (1) chaetae anteriorly and posteriorly of about the same size, not enlarged in caudal segments; (2) oesophageal appendage with tertiary branches; (3) three

**Figure 1.** Habitat of *Hemienchytraeus wuhanensis* sp. nov., Mount Shizi, Huazhong Agriculture University, Wuhan, Hubei Province, China.

pairs of secondary pharyngeal gland ventral lobes in V, VI, VII, small in VII; (4) five pairs of preclitellar nephridia in 5/6–9/10; (5) dorsal vessel originating in clitellum segments; (6) clitellum girdle-shaped; (7) seminal vesicle absent; (8) spermathecae extending to VI–VII, not enlarged.

**Description.** ***Length*** 6.5–9.3 mm (*in vivo*), ***diameter*** 0.3–0.4 mm (*in vivo*) at clitellum. ***Segment number*** 37–42. Two chaetae per bundle throughout, absent in XII in mature specimens. ***Chaetae*** straight with slight proximal bend; in anterior segments, slight distal bend in opposite direction of proximal bend, i.e., chaetae faintly sigmoid; in proximal segments, chaetae distally straight. Chaetae in preclitellar bundles 37.5–42 mm long, diameter 5 mm, 27.5–32.5 mm in postclitellar segments, diameter 5 mm. ***Head pore*** mid-dorsally on prostomium. ***Epidermal gland cells*** gray, three to four transverse rows per segment, the cells nearly rectangular and arranged in regular pattern (Fig. 3E). ***Clitellum*** in XII–1/2XIII, inconspicuous thickening, cells ca 5–9 mm high, girdle-shaped (Fig. 3I, J), hyalocytes and granulocytes in reticulate arrangement with hyalocytes taking larger proportion dorsally (Fig. 3I). ***Body wall*** 25–37.5 mm thick.

***Brain*** about as long as wide (117 mm long, 93 mm wide, *in vivo*), slightly indented anteriorly, deeply incised posteriorly (Figs 2B, 3A). ***Oesophageal appendage*** arising from mid-dorsal region of pharynx in III as an unpaired root with large proximal chamber; following section longer than proximal chamber, with thick, meandering canal; two primary branches, longer than root, with smaller canal; each primary branch bifurcating into two short, secondary branches; each secondary branch bifurcating into four or more tertiary branches, the latter difficult to distinguish. Secondary and tertiary branches of same diameter, thinner than primary branches (Figs 2E, 3B, C). All three pairs of ***pharyngeal glands*** united dorsally, primary ventral lobes in V and VI. Three pairs of secondary pharyngeal gland lobes in V, VI and VII, small in VII (Figs 2D, 3D). ***Dorsal vessel*** from XII–XIII, blood colorless.

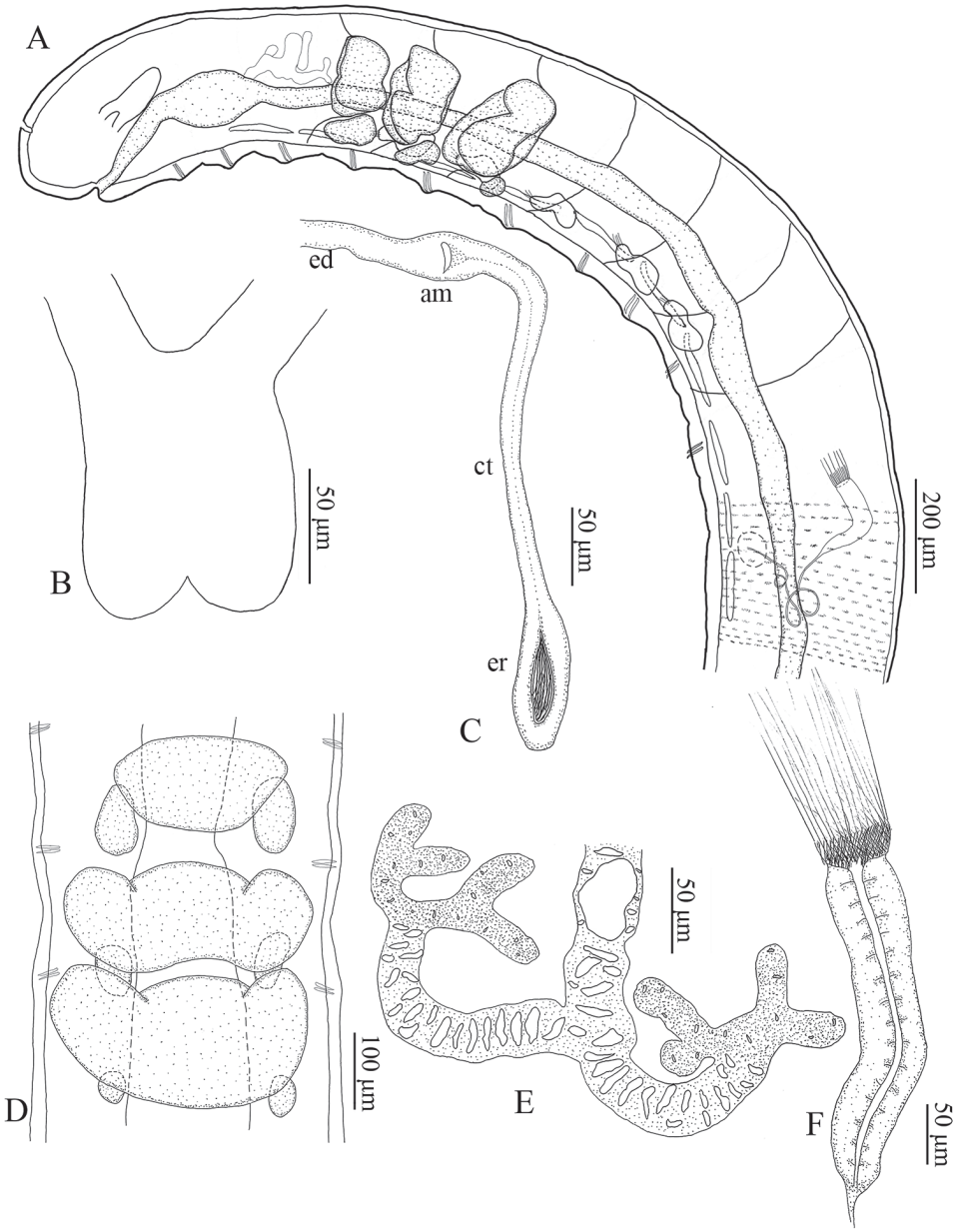


**Figure 2.** *Hemienchytraeus wuhanensis* sp. nov. **A** anterior body region, anterior 13 segments, lateral view, schematic **B** brain **C** spermatheca; am, ampulla; ct, connecting tube; ed, ectal duct; er, ental reservoir **D** pharyngeal glands **E** oesophageal appendage **F** sperm funnel.

Five pairs of preclitellar ***nephridia*** from 5/6 to 9/10 (Fig. 2A); each about 160 mm long and 60 mm wide (*in vivo*). Anteseptale globular, with minute and numerous brownish granules at periphery; funnel orientated obliquely ventrad, with small and

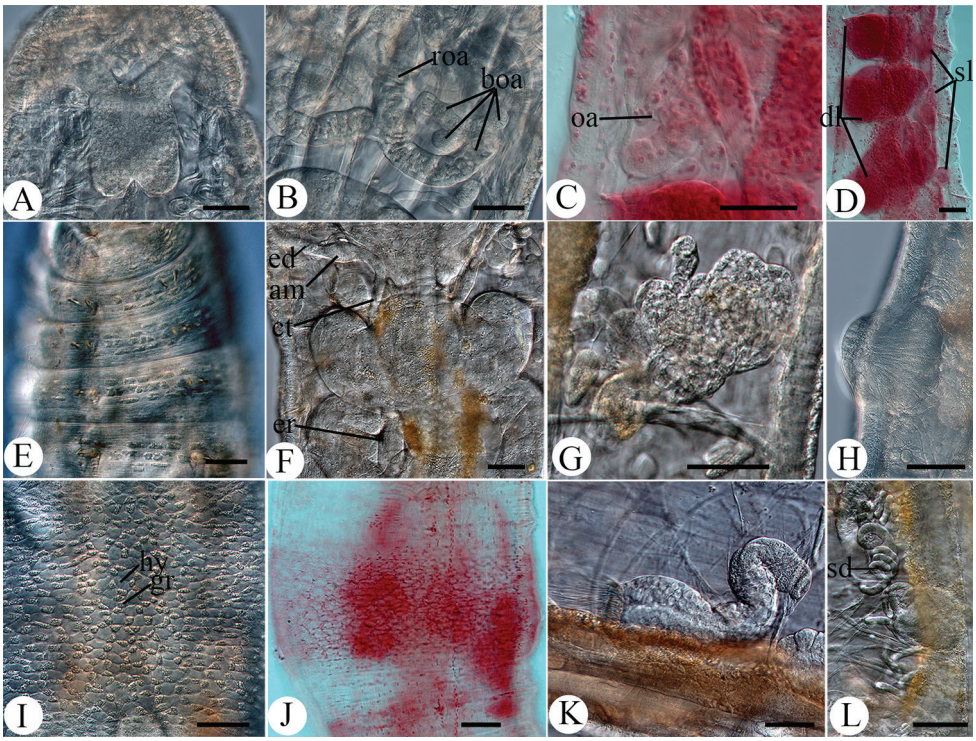


**Figure 3.** Micrographs of *Hemienchytraeus wuhanensis* sp. nov. **A, B, E–I, K, L** *in vivo* **C, D, J** fixed **A** brain **B** dorsal view of oesophageal appendage **C** lateral view of oesophageal appendage **D** pharyngeal glands **E e**pidermal gland cells in II–V ventrally **F** spermathecae and pharyngeal glands **G** nephridia in 7/8, anteseptale bottom-left **H** male glandular bulb, slightly everted **I** dorsal view of clitellum **J** ventral view of clitellum **K** sperm funnel **L** sperm duct and musculature of male copulatory organ Abbreviations: roa, root of oesophageal appendage; boa, branches of oesophageal appendage; oa, oesophageal appendage; sl, secondary pharyngeal gland lobes; dl, dorsal lobes of pharyngeal gland; am, ampulla; ct, connecting tube; ed, ectal duct; er, ental reservoir; hy, hyalocyte; gr, granulocyte; sd, sperm duct. Scale bars: 50 μm.

narrow anterior projection; postseptale elongate, ca twice as long as anteseptale. Efferent duct originating from the middle of the postseptale (Fig. 3G).

***Seminal vesicle*** absent, cysts dorsally in XI. ***Sperm funnels*** cylindrical, tapering distad, well developed, ca 150–250 mm long and 40 mm at collar (*in vivo*). Collar distinct, somewhat narrower than funnel body (Figs 2F, 3K). Spermatozoa ca 140 mm long, heads ca 20 mm long (*in vivo*). Sperm ducts elongate, diameter ca 6 mm, loose or tight coils in XII–XIII (Fig. 3L). ***Male copulatory organs*** with distinct musculature, male glandular body globular, ca 85 μm in diameter (*in vivo*). No accessory copulatory glands (Fig. 3H).

***Spermathecae*** free, not attached to oesophagus. Ectal pores laterally at 4/5, without ectal gland. Ectal ducts ca 400–500 mm long and 20–26 mm wide (*in vivo*), with distinct ampullar dilatation in V. Connecting tube between ampulla proper and ental reservoir thinner than ectal duct, extending into VI or VII, ending in a small, elongately

**Table 1.** List of *Hemienchytraeus* specimens for molecular analyses with collection data and GenBank accession.

| Species | Collection information | Specimen ID | Accession number |
|---|---|---|---|
| *H. wuhanensis*-1 | Mt Luojia, China | HBO201904011 | MW000758 |
| *H. wuhanensis*-2 | Mt Luojia, China | HBO201904012 | MW000759 |
| *H. wuhanensis*-3 | Mt Shizi, China | HBO201904005 | MW000760 |
| *H. wuhanensis*-4 | Mt Shizi, China | HBO201904006 | MW000761 |
| *H. wuhanensis*-5 | Mt Shizi, China | HBO201904007 | MW000762 |
| *H. quadratus*-1 | Mt Hallasan, Korea | 1000 | MG252159 |
| *H. quadratus*-2 | Mt Hallasan, Korea | 991 | MG252158 |
| *H. koreanus*-1 | Mt Hallasan, Korea | 1131 | MG252157 |
| *H. koreanus*-2 | Mt Hallasan, Korea | 1005 | MG252156 |
| *H. koreanus*-3 | Mt Hallasan, Korea | 1004 | MG252155 |
| *H. koreanus*-4 | Mt Hallasan, Korea | 1003 | MG252154 |
| *H. koreanus*-5 | Mt Hallasan, Korea | 1002 | MG252153 |
| *H. jeonjuensis* | Mt Hallasan, Korea | 1115 | MG252152 |

**Table 2.** Genetic distances of four *Hemienchytraeus* species (K2P).

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *H. wuhanensis*-1 | | | | | | | | | | | | |
| 2 | *H. wuhanensis*-2 | 0.000 | | | | | | | | | | | |
| 3 | *H. wuhanensis*-3 | 0.000 | 0.000 | | | | | | | | | | |
| 4 | *H. wuhanensis*-4 | 0.000 | 0.000 | 0.000 | | | | | | | | | |
| 5 | *H. wuhanensis*-5 | 0.000 | 0.000 | 0.000 | 0.000 | | | | | | | | |
| 6 | *H. quadratus*-1 | 0.200 | 0.200 | 0.200 | 0.200 | 0.200 | | | | | | | |
| 7 | *H. quadratus*-2 | 0.198 | 0.198 | 0.198 | 0.198 | 0.198 | 0.007 | | | | | | |
| 8 | *H. koreanus*-1 | 0.070 | 0.070 | 0.070 | 0.070 | 0.070 | 0.216 | 0.216 | | | | | |
| 9 | *H. koreanus*-2 | 0.070 | 0.070 | 0.070 | 0.070 | 0.070 | 0.216 | 0.216 | 0.000 | | | | |
| 10 | *H. koreanus*-3 | 0.070 | 0.070 | 0.070 | 0.070 | 0.070 | 0.216 | 0.216 | 0.000 | 0.000 | | | |
| 11 | *H. koreanus*-4 | 0.070 | 0.070 | 0.070 | 0.070 | 0.070 | 0.216 | 0.216 | 0.003 | 0.003 | 0.003 | | |
| 12 | *H. koreanus*-5 | 0.072 | 0.072 | 0.072 | 0.072 | 0.072 | 0.213 | 0.213 | 0.002 | 0.002 | 0.002 | 0.005 | |
| 13 | *H. jeonjuensis* | 0.219 | 0.219 | 0.219 | 0.219 | 0.219 | 0.189 | 0.191 | 0.214 | 0.214 | 0.214 | 0.211 | 0.211 |

ellipsoid ental reservoir of 88–128 μm length and 30–50 μm width (*in vivo*), empty or with spermatozoa (Figs 2C, 3F). One mature egg or 3–4 immature eggs at a time.

## Molecular results

COI sequences of five paratype specimens of *H. wuhanensis* sp. nov. were successfully acquired and submitted to GenBank with accession numbers. This is the fourth species of *Hemienchytraeus* of which DNA sequences are available (Table 1), the other three being *H. quadratus*, *H. koreanus*, and *H. jeojunensis* Dózsa-Farkas & Hong, 2010, all from South Korea. Clear genetic gaps were observed among the four species with high interspecific distances (7.0–21.9%) and low intraspecific distances (0%) among *H. wuhanensis* sp. nov. specimens based on the K2P distances of COI sequences (Table 2). Interestingly, among the three species from South Korea, the one with lowest genetical distance to *H. wuhanensis* sp. nov., *H. koreanus*, is also the one which is most similar morphologically to the new species (see below).

## Remarks

Three non-sexual characters have been shown to be very useful for the distinction of *Hemienchytraeus* species: oesophageal appendage (branching pattern, relative branch length), secondary pharyngeal gland lobes (number, position, size), and preclitellar nephridia (number, position) (Schmelz et al. 2009). Indeed, these three characters in *H. wuhanensis* suffice to distinguish it from all other species, even from those with an incomplete description, because details of the oesophageal appendage are known in all species, the only exception being *H. brasiliensis* (Cognetti, 1900), a species of uncertain identity (*incertae sedis*) according to Schmelz and Römbke (2005). Further useful characters include the origin of the dorsal blood vessel, presence/absence of a seminal vesicle, shape and size of spermathecae, sperm funnels and male glandular bulbs, and distribution pattern of clitellar gland cells; the latter is fully known only in recently described species.

Considering the three above-mentioned non-sexual diagnostic characters, the new species is most similar to *H. loksai* Dózsa-Farkas, 1989, which also has an oesophageal appendage with tertiary branches, three pairs of secondary pharyngeal gland lobes in V, VI, VII, and five pairs of preclitellar nephridia, from 5/6 to 9/10. However, in *H. loksai* the secondary pharyngeal glands increase in size from IV to VII. The species was described from Ecuador and has been recorded from China (Xie et al. 1999). Further conspicuous differences of *H. loksai* from the new species include larger body size (length >12 mm, 49–55 segments), a postclitellar origin of the dorsal blood vessel, larger spermathecae (extending to IX–X), very large sperm funnels (up to 800–900 μm long), and a huge seminal vesicle (extending into XIV–XVII).

One more species of *Hemienchytraeus* has oesophageal appendages with tertiary branches, i.e., *H. brachythecus* Xie et al., 1999. This species is also similar to the new species in the absence of a seminal vesicle. Conspicuous differences of *H. brachythecus* include a very short spermatheca, confined to V, two pairs of secondary pharyngeal gland lobes in V and VI, and first pair of preclitellar nephridia in 6/7.

Three pairs of secondary pharyngeal gland lobes are also known in *H. koreanus* Dózsa-Farkas & Hong, 2010, and in *H. siljae* Schmelz & Römbke, 2005. *H. koreanus* resembles the new species also in the position of the preclitellar nephridia (5/6–9/10) and in a girdle-shaped clitellum. Conspicuous differences of *H. koreanus* include a postclitellar origin of the dorsal blood vessel, large spermathecae, and the presence of a seminal vesicle.

*H. siljae* resembles the new species in several characters, for example the girdle-shaped clitellum, the absence of a seminal vesicle, and the approximate shape and size of spermathecae and sperm funnels. Conspicuous differences include a more posterior origin of the dorsal blood vessel (XIV), four pairs of preclitellar nephridia, from 6/7 to 9/10, and an oesophageal appendage with three elongate secondary branches on each side, without tertiary branches.

A comparison of these four species with the new one is presented in Table 3.

With the description of *H. wuhanensis* sp. nov., there are now seven species of *Hemienchytraeus* known from China. Two of them were originally described from China

**Table 3.** Comparison of *H. wuhanensis* sp. nov. with similar species.

| | ***H. wuhanensis* sp. nov.** | ***H. brachythecus* Xie et al., 1999** | ***H. siljae* Schmelz et al., 2005** | ***H. loksai* Dózsa-Farkas, 1989** | ***H. koreanus* Dózsa-Farkas & Hong, 2010** |
|---|---|---|---|---|---|
| Secondary pharyngeal gland lobes | 3 pairs, V–VII | 2 pairs, V–VI | 3 pairs, V–VII | 3 pairs, V–VII | 3 pairs, V–VII |
| Oesophageal appendage | 4 or more tertiary branches | 3–4 tertiary branches | 4–5 elongate secondary branches | 3–4 tertiary branches | 5–6 secondary branches |
| Preclitellar nephridia | 5; 5/6–9/10 | 5; 6/7–10/11 | 4; 6/7–9/10 | 5; 5/6–9/10 | 5; 5/6–9/10 |
| Sperm funnel: shape; length:width ratio | Cylindrical; 4–6:1 | Subspherical; 1.6–2:1 | Cone-shaped; 4–6:1 | Cone-shaped; 9:1 | Cone-shaped; 5–6:1 |
| Spermathecae, extension | VI–VII | V | VI–VIII | IX–X | VIII–X |
| Seminal vesicle | Absent | Absent | Absent | XII–XIV | XII–XIII |
| Epidermal gland cells | 3–4 rows per segment | Scarce | 4–5 rows in preclitellar segments | 6–8 rows per segment | 3–4 rows per segments |

and have not been recorded elsewhere: *Hemienchytraeus planisetosus* Xie et al., 1999 and *Hemienchytraeus brachythecus* Xie et al., 1999. The other four species were originally described from different countries, and the records from China require confirmation, for various reasons.

The record of *Hemienchytraeus stephensoni* Cognetti, 1927, from Hunan Province (Xie et al. 1999) was rejected by Schmelz and Collado (2007), after a type-based revision of this nominal species (Schmelz and Collado 2007), which narrowed the range of variation of taxonomically important characters. *Hemienchytraeus stephensoni* sensu Xie et al. (1999) may in fact be a species new to science. *Hemienchytraeus stephensoni* was originally described from India as *Enchytraeus cavicola* Stephenson, 1924; see Schmelz and Collado (2007) for the nomenclatural history.

*Hemienchytraeus bifurcatus* Nielsen & Christensen, 1959 originally described from Denmark, has been considered a "*species inquirenda*" (Schmelz and Römbke 2005), because the original description is incomplete with respect to secondary pharyngeal gland lobes, preclitellar nephridia, and details of the clitellum. A validation of *H. bifurcatus* is difficult because type material is lost, and efforts to obtain fresh material at the type locality have so far been unsuccessful (Schmelz and Römbke 2005). Hence, the records of this species from China (Liang and Xie 1992; Wang and Liang 2002) cannot be confirmed; those specimens may just as well belong to a new species.

The redescription of *H. loksai* by Xie et al. (1999) based on material from Hunan Province, China, agrees with the original description in conspicuous details (e.g., size of seminal vesicle and sperm funnels) but lacks information on the secondary pharyngeal gland lobes; furthermore, the first preclitellar nephridia are in 6/7, not in 5/6 as originally described. Material of *H. loksai* sensu Xie et al. (1999) should be reinvestigated to confirm the species identity of the specimens.

Finally, *H. theae* Prabhoo, 1961 described from India, and recorded from China by Liang and Xie (1992), was originally insufficiently described: secondary pharyngeal gland lobes, number and position of nephridia, details of the clitellum, and origin of the dorsal blood vessel are unknown. Reinvestigation of the type material present at the Zoological Survey of India (Prabhoo 1961) and comparison with the material un-

derlying the record of Liang and Xie (1992) would be necessary to confirm the species identity of the Chinese specimens.

Despite these taxonomic uncertainties, the presence of at least seven species of *Hemienchytraeus* in China is beyond doubt. Actually, many more species of *Hemienchytraeus* are to be expected in this country, in view of the preference for tropical or subtropical soils of this globally distributed genus.

## Acknowledgements

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# A new species of the genus *Opisa* Boeck, 1876 (Crustacea, Amphipoda, Opisidae) and a new record for *Opisa takafuminakanoi* from the East Sea, South Korea

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## Abstract

Two species of the opisid genus *Opisa* have been collected from the East Sea of South Korea, one of them described as *Opisa parvimana* **sp. nov.**. The new species, *O. parvimana* **sp. nov.** is similar to *O. odontochela*; however, it can be clearly distinguished from this species because it has 12 blunt robust setae in the palm of gnathopod 1. The other collected species, *Opisa takafuminakanoi* Narahara-Nakano, Kakui & Tomikawa, 2016 is previously known from Japanese waters (southeast of Akkeshi Bay, Hokkaido). Both species are illustrated and compared to related species. A key to *Opisa* species is also provided.

## Keywords

Identification key, Lysianassoidea, *Opisa parvimana* sp. nov., parasitic amphipod, taxonomy

## Introduction

The family Opisidae was first established by Lowry and Stoddart (1995) for a small group of lysianassoid amphipods that currently includes 19 species in four genera (Narahara-Nakano et al. 2016; Horton et al. 2020). They are mainly known from

the North Pacific Ocean, North Atlantic Ocean, and Mediterranean Sea (Stoddart and Lowry 2010). Most opisids species are considered ectoparasitic in fish (Vader and Romppainen 1985; Bousfield 1987; Stoddart and Lowry 2010), attracted to the smell of the fish, to which they attach instead of scavenging, because they do not have the mouthpart structure of a scavenger (Stoddart and Lowry 2010). Parasitic amphipods are typically found on slow-moving, slow-growing benthic sharks and bony fishes in cold or deep waters; as a group, rockfish, sculpins, goosefishes, and flatfishes may be described as ambush predators (Vader and Romppainen 1985; Bousfield 1987).

The genus *Opisa* Boeck, 1876 includes a total of four species: *O. eschrichtii* (Krøyer, 1842), *O. odontochela* Bousfield, 1987, *O. tridentata* Hurley, 1963, and *O. takafuminakanoi* Narahara-Nakano, Kakui & Tomikawa, 2016, with the last one reported in Japan (Narahara-Nakano et al. 2016). In this study, we report an additional new species and a newly recorded species of *Opisa* from South Korea through illustrations and text descriptions. This study also provides a key to *Opisa* species around the world.

## Materials and methods

The material examined was collected with a fishing net from subtidal waters of the Namae Port, East Sea, South Korea. Specimens were fixed in 70–80% ethanol and dissected in glycerol on Cobb's aluminum hole slides. Examinations were performed using a stereoscope (Olympus SZX 10) and a compound microscope (Olympus BX 51), and the drawings and measurements were made with the aid of a drawing tube. The body length was measured from the tip of rostrum to the end of the telson, along the dorsal parabolic line of the body. Nomenclature of the term 'seta' follows Watling (1989), Garm and Watling (2013). Terminology of the setae of the mandibular palp follows G. Karaman (1969, 1971) and Lowry and Stoddart (1993). Type specimens are deposited at the National Institute of Biological Resources (**NIBR**), Incheon, South Korea and the Marine Amphipoda Resources Bank of Korea (**MARBK**), Cheonan, South Korea.

## Taxonomy

**Family Opisidae Lowry & Stoddart, 1995**
Korean name: Jib-ge-son-gin-pal-yeop-sae-u-gwa, new

**Genus *Opisa* Boeck, 1876**
Korean name: Jib-ge-son-gin-pal-yeop-sae-u-sok, new

**Type species.** *Opisa eschrichtii* Krøyer, 1842

***Opisa parvimana* sp. nov.**
http://zoobank.org/104C5232-D9FA-4CD4-9E9C-110894AF0FAC
Korean name: Jag-eun-jib-ge-son-gin-pal-yeop-sae-u, new

**Type material.** ***Holotype***, male, 8.3 mm, MARBK-300 and female, 7.2 mm, MARBK-301, South Korea: Namae Port, Yangyang-gun, Gangwon-do, 37°56'32"N, 128°47'12"E, Y.H. Kim, 21 December 2007. ***Paratypes*** (one male, one female, MARBK-302), same station data as holotype.

**Diagnosis.** Lateral cephalic lobe subacutely projecting. Mouthparts forming quadrate bundle. Antenna 1, callynophore well developed; flagellum short, 3–5 articles with calceoli in male. Antenna 2, flagellum elongated, with calceoli in male. Upper lip, epistome normal. Mandible, molar setose, left lacinia mobilis blunt. Maxilla 1, outer plate with 11 dentate spine-teeth in an 8/3 crown arrangement. Gnathopod 1, palm straight, armed with a row of 12 blunt robust setae and 1 slender seta, defined by short and subacute process. Uropods 1–2, each ramus with distinct notch with inserted robust setae. Uropod 3, outer ramus biarticulate, longer than inner ramus. Telson cleft.

**Description.** *Holotype*, *adult male*: body (Figs 1A, 2A) dorsally smooth, 8.3 mm long. Head, lateral cephalic lobe subacute, triangular, slightly concave ventrally; eye large, reniform, black. Epimeron 1 posterior margin smooth and concave; epimeron 2 posteroventral corner right angled; epimeron 3 posteroventral corner rounded. Urosomite 1 with mid-dorsal depression and dorsal carina.

Antenna 1 (Fig. 2B) short, 1.29× head; peduncular article 1 much longer than peduncular articles 2–3 combined, with a row of 10 penicillate setae dorsally; length ratio of peduncular articles 1–3 = 1.00 : 0.28 : 0.17; flagellum 9-articulate, 0.86× shorter than peduncular articles, with 2-field callynophore, calceoli on flagellum articles 3–5; accessory flagellum 5-articulate, article 1 rather elongated.

Antenna 2 (Fig. 2C) slender, elongated, 0.61× body; peduncular article 4 shorter than peduncular article 5, with a row of short setae dorsally, 2 penicillate setae and unequal simple setae anterodistally, 5 penicillate setae ventrally; peduncular article 5 with simple setae dorsally, 4 penicillate setae ventrally; flagellum 55-articulate, calceoli on flagellum articles, some articles missing the calceoli.

Lower lip (Fig. 2D), inner lobes distinct, oval, with pubescence distally; outer lobe with pubescence on distal and medial margins; mandibular lobes elongated.

Left mandible (Fig. 2E), incisor simple, smooth, with a blunt tooth; lacinia mobilis blunt; accessory setal row with 3 robust setae; molar setose, not triturative, as a rounded lobe.

Right mandible (Fig. 2F), incisor smooth, with a blunt tooth; lacinia mobilis absent; accessory setal row with 3 robust setae; palp 3-articulate, attached proximal to molar; article 1 unarmed, short, 0.58× article 3; article 2 longest, with 7 A2-setae; article 3 weakly falcate, 0.88× article 2, with 11 D3-setae and 3 E3-setae.

Maxilla 1 (Fig. 2G), inner plate slender, subrectangular, with 1 pectinate and 1 simple setae apically and setules on outer margin; outer plate with 11 dentate spine-

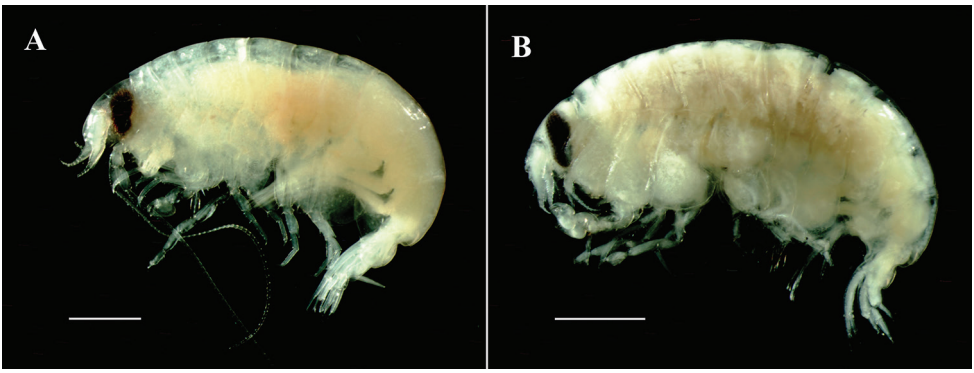


**Figure 1.** *Opisa parvimana* sp. nov. **A** adult male, MARBK-300, 8.3 mm, habitus **B** adult female, MARBK-301, 7.2 mm, habitus. Scale bars: 1.0 mm (**A, B**).

teeth; palp biarticulate, proximal article short, distal article expanded, with 2 slender setae and 6 blunt robust setae apically.

Maxilla 2 (Fig. 2H), inner plate slender, slightly shorter than outer, with 11 apical setae and 1 pectinate seta mediodistally, medial margin with pubescence; outer plate 1.08× longer than inner one, with 13 simple setae distally.

Maxilliped (Fig. 2I), inner plate rectangular, with 3 pectinate setae medially, apical margin with 2 unequal simple setae and 2 blunt robust setae; outer plate moderately expanded, not reaching distal end of article 3 of palp, with 8 blunt robust setae on inner margin and 7 short simple setae medially; palp 4-articulate, article 1 slightly shorter than article 2, with 1 simple seta on inner margin; article 2 with 7 simple setae on inner margin; article 3 slightly shorter than article 2, with simple setae on inner and distal margins; article 4 falcate, 0.47× shorter than article 3.

Gnathopod 1 (Fig. 2J), coxa rounded anterodistally; basis subrectangular, bulge anterodistally; ischium elongated, 0.37 as long as basis, with 1 simple seta posteriorly; carpus unarmed, slightly expanded posteriorly; propodus subequal in length to carpus, ovate, rounded and smooth posteriorly, palm straight, armed with a row of 12 blunt robust setae and 1 slender seta, defined by short and subacute process, with 2 robust setae subapically; dactylus falcate, stout, inner margin evenly dentate.

Gnathopod 2 (Fig. 3A), coxa subrectangular, slightly widening distally, width 0.49× length; basis slender, elongated, with 1 simple seta anterodistally; ischium elongated, 0.74× carpus, anterior and posterior margins each with 2 simple setae; merus 0.60× ischium, with patch of setules posteriorly and 4 unequal simple setae posterodistally; carpus, posterodistal margin surface with patch of setules, with unequal setae each distal margins, 0.57× basis, posterior margin slightly convex; propodus short, length 2.00× width, subquadrate, surface covered by setules, with cluster of setae anterodistally, palm slightly oblique, with setules, defined by 1 tiny blunt seta posterodistally; dactylus falcate, short, with accessory tooth.

Pereopod 3 (Fig. 3B), coxa similar to that of gnathopod 2, but slightly more widening distally, width 0.49× length; basis slender, with 2 simple setae posterodistally; is-

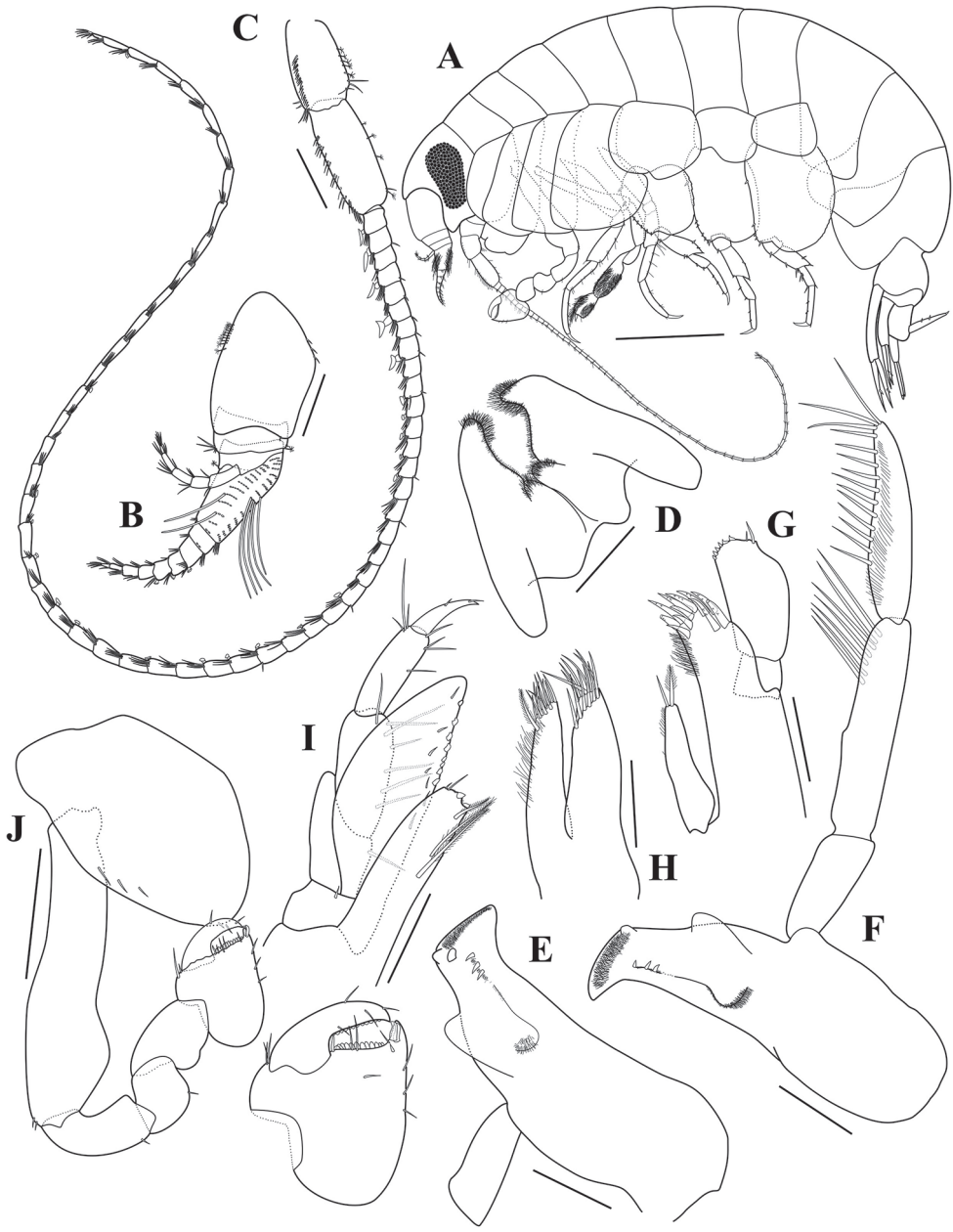


**Figure 2.** *Opisa parvimana* sp. nov. holotype, adult male, MARBK-300, 8.3 mm **A** habitus **B** antenna 1 **C** antenna 2 **D** lower lip **E** left mandible **F** right mandible **G** maxilla 1 **H** maxilla 2 **I** maxilliped **J** gnathopod 1. Scale bars: 1.0 mm (**A**), 0.2 mm (**B, C**), 0.1 mm (**D–I**), 0.4 mm (**J**).

chium short, 0.19× basis, with 2 unequal simple setae posterodistally; merus subequal in length to carpus, slightly produced anterodistally, with 1 simple seta anterodistally and 6 unequal simple setae; carpus subrectangular, with 1 simple seta anterodistally,

unequal setae posteriorly; propodus subrectangular, slightly shorter than carpus, with long simple setae posteriorly; dactylus falcate, with 1 penicillate seta anteriorly.

Pereopod 4 (Fig. 3C) similar to pereopod 3 except coxa broadened, posterior margin excavate, posterodistal lobe produced, truncate, corner rounded.

Pereopod 5 (Fig. 3D), coxa large, with rounded corners, subquadrate, hind lobe margin angled distally, width subequal to length; basis subcircular, width subequal to length, expanded posteriorly, margin serrate, posteroventral lobe broadly rounded, anterior margin with a row of robust setae; merus expanded posteriorly, anterior margin with 4 simple setae and 3 robust setae, posterior margin with 3 robust setae; carpus 0.56× merus, anterior margin with 2 robust setae and 3 robust setae distally, posterior margin with 1 robust seta distally; propodus rectangular, 1.70× carpus, anterior margin with 3 robust setae; dactylus falcate, with 1 penicillate seta posteriorly.

Pereopod 6 (Fig. 3E), coxa bilobate, anterior lobe small, posterior lobe roundly produced ventrally; basis subquadrate, posterior margin serrate, posteroventral lobe broadly rounded, anterior margin slightly concave, with 7 robust setae; merus expanded posteriorly, anterior margin with 2 long simple and 3 small robust setae, posterior margin with 2 robust setae; carpus 1.36× merus, anterior margin with 2 robust setae and 3 robust setae distally, posterior margin with 1 robust seta distally; propodus rectangular, 1.43× carpus, anterior margin with 3 clusters of 2 robust setae and 1 robust seta distally; dactylus falcate, with 1 penicillate seta posteriorly.

Pereopod 7 (Fig. 3F) similar to pereopod 6, but coxa unilobate; basis much broader than that of pereopod 6, posterior margin broadly expanded.

Uropod 1 (Fig. 3G), peduncle subrectangular, 1.38× outer ramus, with a row of 5 dorsolateral, 2 dorsomedial, and 1 apicolateral robust setae; each ramus with distinct notch with inserted robust setae; outer ramus subequal in length to inner one, both rami each with 1 dorsolateral and 1 dorsomedial robust setae.

Uropod 2 (Fig. 3H), peduncle subequal in length to both rami, with 4 dorsolateral and 3 medial robust setae; each ramus with distinct notch with inserted robust setae; outer ramus subequal in length to inner one, both rami each with 1 dorsolateral and 1 dorsomedial robust setae.

Uropod 3 (Fig. 3I), peduncle short, 0.58× outer ramus, with 2 ventrodistal, 3 dorsomedial, and 1 dorsolateral robust setae; outer ramus biarticulate, 1.06× inner ramus, proximal article with 6 long plumose setae along inner margin and 2 robust setae laterally, each margin with 1 robust seta distally; distal article short, 0.26× proximal one; inner ramus slightly exceed base of distal article of outer ramus, outer margin with a row of 7 plumose setae, inner margin unarmed.

Telson (Fig. 3J) elongated, length 2.05× width, cleft 84% of its length, each lobe with 2 dorsolateral robust setae, 1 robust seta and 1 penicillate seta apically.

*Paratype*, *adult female*: body (Figs 1B, 4A) about 7.2 mm long. Coxa 1 less anteriorly expanded than that of male.

Antenna 1 (Fig. 4B) stout, similar to that of male except peduncular article 1 with 6 penicillate setae dorsally; flagellum 8-articulate, calceoli absent; accessory flagellum 6-articulate, article 1 not elongated.

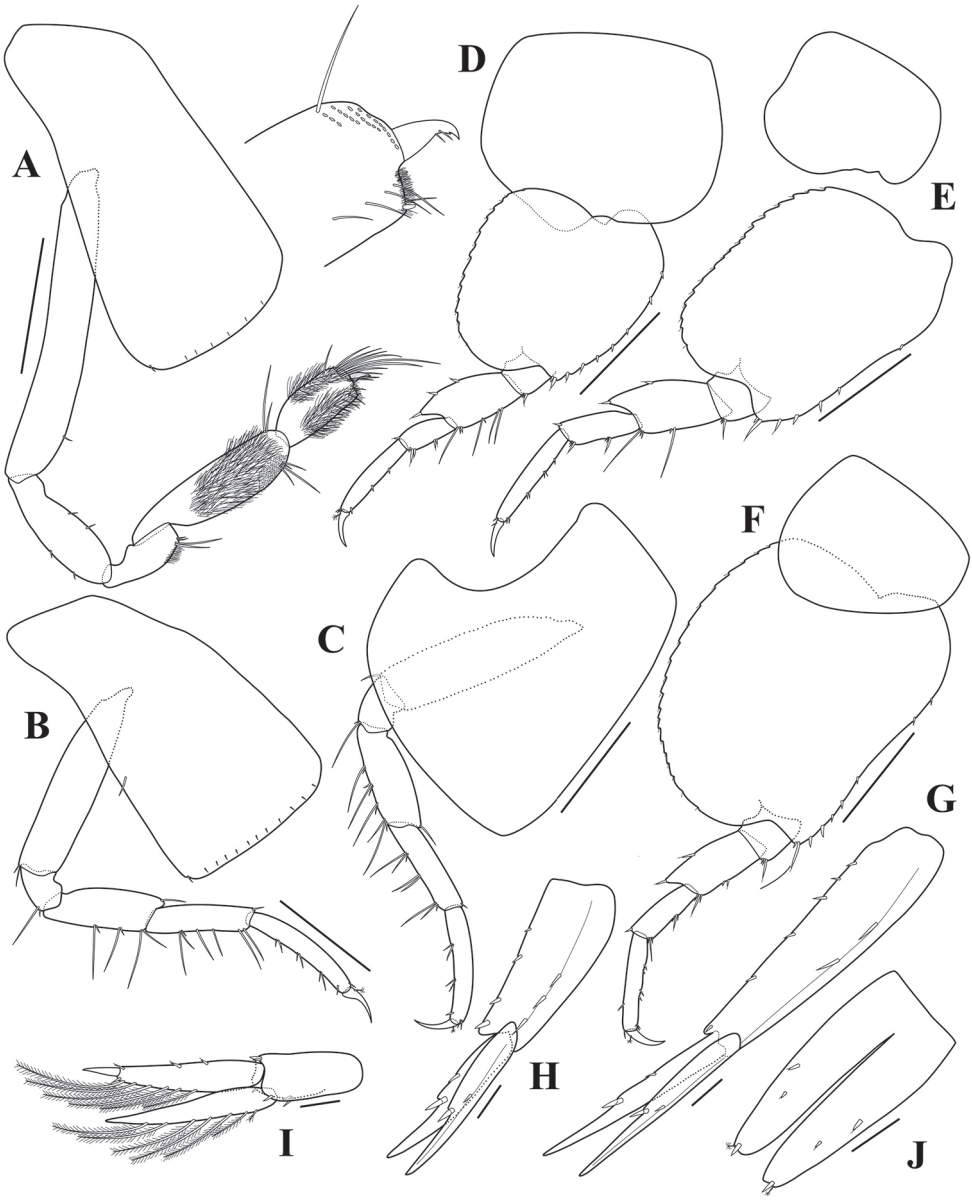


**Figure 3.** *Opisa parvimana* sp. nov. holotype, adult male, MARBK-300, 8.3 mm **A** gnathopod 2 **B** pereopod 3 **C** pereopod 4 **D** pereopod 5 **E** pereopod 6 **F** pereopod 7 **G** uropod 1 **H** uropod 2 **I** uropod 3 **J** telson. Scale bars: 0.4 mm (**A–G**), 0.1 mm (**H–J**).

Antenna 2 (Fig. 4C) slender, much shorter than that of male, peduncular articles 3–5 shorter than those of male; flagellum 7-articulate, calceoli absent.

**Etymology.** The species name is derived from the Latin *parvus* (=small) and *manus* (=hand) with reference to the relatively small propodus of the gnathopod 1.

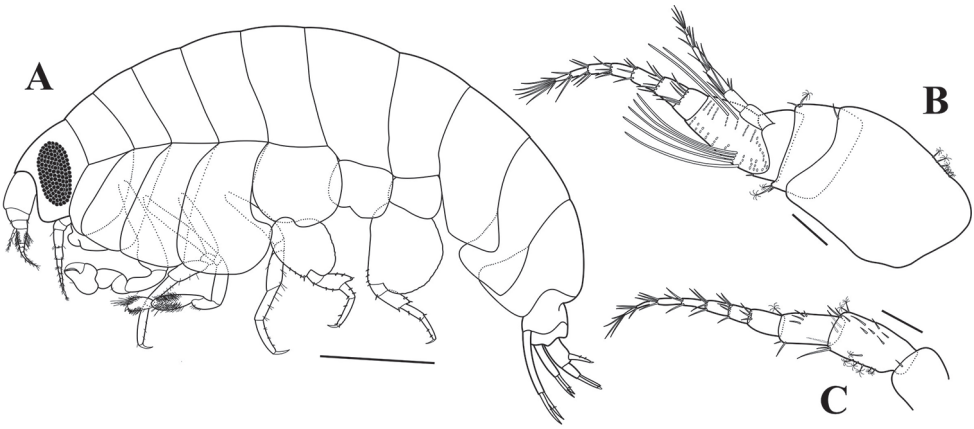


**Figure 4.** *Opisa parvimana* sp. nov. paratype, adult female, MARBK-301, 7.2 mm **A** habitus **B** antenna 1 **C** antenna 2. Scale bars: 1.0 mm (**A**), 0.1 mm (**B, C**).

**Remarks.** The genus *Opisa* Boeck, 1876 is similar to the genera *Cheirimedon* Stebbing, 1888, *Normanion* Bonnier, 1893, *Podoprionella* G.O. Sars, 1895, and *Podoprionides* Walker, 1906 in having deep coxal plates, a bilobate telson, small modification or reduction of mandible and maxilliped palps, and distinctly biarticulate outer ramus of uropod 3. However, the genus *Opisa* is easily distinguished from these genera by the following features: 1) enlarged gnathopod 1, strongly subchelate or cheliform; 2) mandibular molar very reduced or even missing; and 3) maxilliped, broadened outer plate and reduced palp (Bousfield 1987).

*Opisa parvimana* sp. nov. is similar to *O. odontochela* Bousfield, 1987 based on the following characteristics: 1) gnathopod 1 with upwardly directed dactylus; 2) gnathopod 1, palm with lined robust setae; 3) gnathopod 2 with single palmar robust seta; and 4) uropods 1 and 2, rami with a robust seta on mid-dorsal margin. However, the new species differs from *O. odontochela* in the following characteristics (compared with the characteristics of *O. odontochela* in parentheses): 1) gnathopod 1, palm with 12 blunt robust setae (vs. about 24-toothed rods); 2) uropod 3, margins of rami with robust setae and plumose setae (vs. margins unarmed).

**Distribution.** South Korea (East Sea).

***Opisa takafuminakanoi*** **Narahara-Nakano, Kakui & Tomikawa, 2016**
Korean name: Keun-jib-ge-son-gin-pal-yeop-sae-u, new

*Opisa takafuminakanoi* Narahara-Nakano, Kakui & Tomikawa, 2016: 335, figs 1,2.

**Material examined.** Male, 8.8 mm, NIBRIV0000880624 and female, 8.7 mm, NIBRIV0000880625, South Korea: Namae Port, Yangyang-gun, Gangwon-do, 37°56'32"N, 128°47'12"E, Y.H. Kim, 21 December 2007. The remaining specimens (two males, three females), same station data as description specimens.

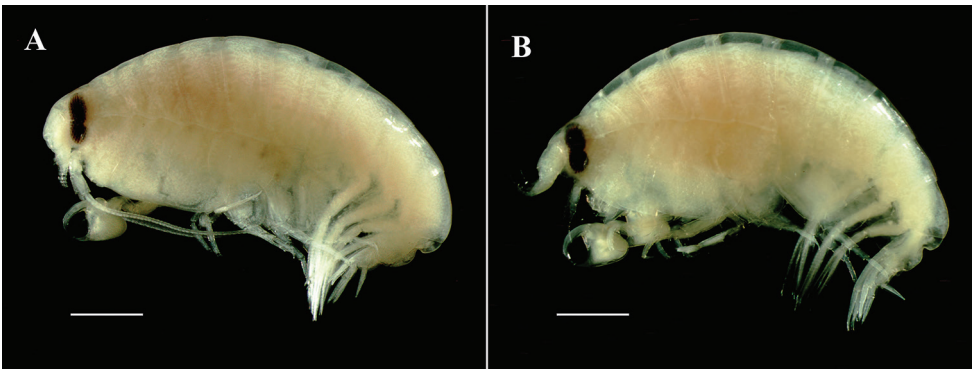


**Figure 5.** *Opisa takafuminakanoi* Narahara-Nakano, Kakui & Tomikawa, 2016 **A** adult male, NIBRIV0000880624, 8.8 mm, habitus **B** adult female, NIBRIV0000880625, 8.7 mm, habitus. Scale bars: 1.0 mm (**A, B**).

**Diagnosis.** Lateral cephalic lobe rounded. Mouthparts forming subquadrate bundle. Antenna 1, callynophore well developed; flagellum short, calceoli absent. Antenna 2, flagellum elongated, calceoli absent. Upper lip, epistome normal. Mandible, molar setose, left lacinia mobilis vestigial. Maxilla 1, outer plate with 11 dentate spine-teeth in an 8/3 crown arrangement. Gnathopod 1 enlarge, palm strongly concave, with unequal simple setae, defined by 2 robust setae subapically. Uropods 1–2, each ramus without notch. Uropod 3, outer ramus biarticulate, longer than inner ramus. Telson cleft.

**Description.** *Adult male*: body (Figs 5A, 6A) 8.8 mm long, dorsally smooth. Lateral cephalic lobe rounded. Eye large, reniform, black. Epimeron 1 with rounded-quadrate posteroventral corner; epimeron 2 posteroventral corner right angled; epimeron 3 subquadrate. Urosomite 1 with mid-dorsal depression and dorsal carina.

Antenna 1 (Fig. 6B) short, 1.71× head; peduncular article 1 much longer than peduncular articles 2–3 combined, with a row of 9 penicillate setae dorsally; length ratio of peduncular articles 1–3 = 1.00 : 0.31 : 0.25; flagellum 10-articulate, 0.86× shorter than peduncular articles, with 2-field callynophore, calceoli absent; accessory flagellum 5-articulate, article 1 slightly elongated.

Antenna 2 (Fig. 6C) slender and elongated; peduncular article 4 shorter than peduncular article 5, with a row of small setae dorsally, 2 penicillate setae dorsodistally, 6 simple setae distally, 2 penicillate setae and 2 unequal simple setae ventrodistally; peduncular article 5 rectangular, with a row of simple setae dorsally and a cluster of long simple setae, 2 penicillate setae ventrally, 1 long simple and 1 penicillate setae ventrodistally; flagellum 48-articulate, calceoli absent.

Gnathopod 1 (Fig. 6D) strongly chelate, enlarge; coxa rounded anterodistally; basis subrectangular, slightly bulge distally; ischium 0.32 as long as basis, unarmed; carpus 1.25× ischium; propodus enlarge, strong, developed posteriorly, palm strongly concave, with unequal simple setae on palmar margin, defined by 2 robust setae subapically, 1.80× carpus; dactylus stout, strongly curved.

Gnathopod 2 (Fig. 6E), coxa (Fig. 6F) subrectangular, slightly widening distally, width 0.51× length; basis slender, elongated, with 1 simple anterodistal seta; ischium

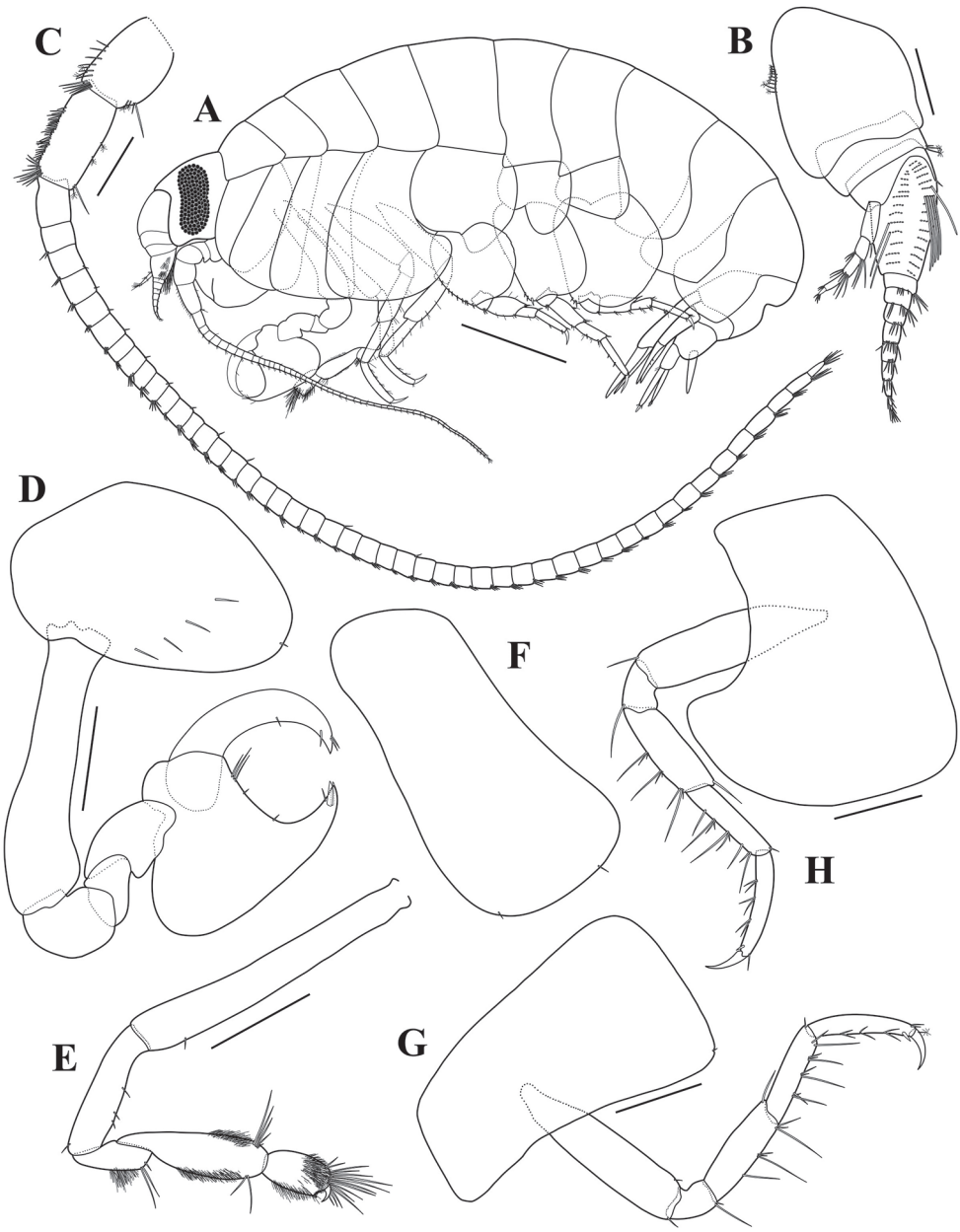


**Figure 6.** *Opisa takafuminakanoi* Narahara-Nakano, Kakui & Tomikawa, 2016, adult male, NIBRIV0000880624, 8.8 mm **A** habitus **B** antenna 1 **C** antenna 2 **D** gnathopod 1 **E** gnathopod 2 **F** coxa 2 **G** pereopod 3 **H** pereopod 4. Scale bars: 1.0 mm (**A**), 0.2 mm (**B, C**), 0.4 mm (**D–H**).

elongated, slightly shorter than carpus, with 3 anterior and 1 posterodistal setae; merus 0.58× ischium, with patch of setules posteriorly and 3 unequal simple setae posterodistally; carpus elongated, 0.49× basis, anterior and posterior margins covered with setules and with distal unequal group of setae, posterior margin slightly convex; propodus

short, length 1.86× width, subquadrate, surface covered by setules, with cluster of setae anterodistally, palm oblique, defined by 2 tiny blunt robust setae posterodistally; dactylus falcate, short.

Pereopod 3 (Fig. 6G), coxa similar to that of gnathopod 2, but slightly more widening distally, width 0.60× length; basis slender, with 2 simple setae distally; ischium short, 0.25× basis, with 2 unequal simple setae posterodistally; merus subequal in length to carpus, slightly produced anterodistally, with 1 simple anterodistal seta and 7 unequal simple setae posteriorly; carpus subrectangular, with 1 simple anterodistal seta, 3 clusters of unequal posterior setae, and 2 simple setae posterodistally; propodus subrectangular, subequal in length to carpus, with a paired setae on posterior margin and 1 robust seta posterodistally; dactylus falcate, with 1 penicillate seta anteriorly.

Pereopod 4 (Fig. 6H) similar to pereopod 3 except coxa broadened, posterior margin excavate, posterodistal lobe produced, truncate, corner rounded.

Pereopod 5 (Fig. 7A), coxa large, with rounded corners, bilobate, posteroventral lobe developed, width 1.24× length; basis subcircular, length 0.90× width, posteriorly expanded, margin serrate, posteroventral lobe broadly rounded, anterior margin with a row of robust setae; merus expanded posteriorly, anterior margin with 3 simple setae and 4 robust setae, posterior margin with 3 simple setae; carpus 0.62× merus, anterior margin with 2 robust setae and 1 simple seta distally, posterior margin with 1 robust seta distally; propodus rectangular, 1.50× carpus, anterior margin with 2 robust setae; dactylus falcate, with 1 penicillate seta posteriorly.

Pereopod 6 (Fig. 7B), coxa bilobate, anterior lobe small, posterior lobe strongly protruding downward; basis subquadrate, posterior margin weakly serrate, posteroventral lobe broadly rounded, anterior margin slightly concave, with 6 robust setae; merus slightly expanded posteriorly, anterior margin with 2 long simple setae and 1 small robust seta, posterior margin with 2 robust setae; carpus 0.75× merus, anterior margin with 3 simple setae and 3 robust setae, posterior margin with 1 robust seta distally; propodus rectangular, 1.42× carpus, anterior margin with 2 robust setae and 1 simple seta distally; dactylus falcate, with 1 penicillate seta posteriorly.

Pereopod 7 (Fig. 7C) similar to pereopod 6, but coxa unilobate; basis much broader than that of pereopod 6, posterior margin broadly expanded.

Uropod 1 (Fig. 7D), peduncle subrectangular, 0.86× outer ramus, with a row of dorsolateral robust setae, 3 dorsomedial and 1 apicolateral robust setae; inner ramus with 2 lateral and 1 medial robust setae, outer ramus slightly longer than inner one, with 2 lateral robust setae.

Uropod 2 (Fig. 7E), peduncle slightly longer than outer ramus, with 3 dorsolateral and 2 dorsomedial robust setae; inner ramus unarmed, unconstricted, subequal in length to outer one; outer ramus with 1 lateral robust seta.

Uropod 3 (Fig. 7F), peduncle short, 0.61× outer ramus, with 2 dorsomedial, 1 dorsolateral, and 3 ventrodistal robust setae; outer ramus biarticulate, 1.09× inner ramus, proximal article with 5 plumose setae along inner margin and 1 lateral robust seta, each margin with 1 robust seta distally; distal article short, 0.19× proximal one; inner ramus nearly reach base of distal article of outer ramus, outer margin with 9 plumose setae, inner margin unarmed.

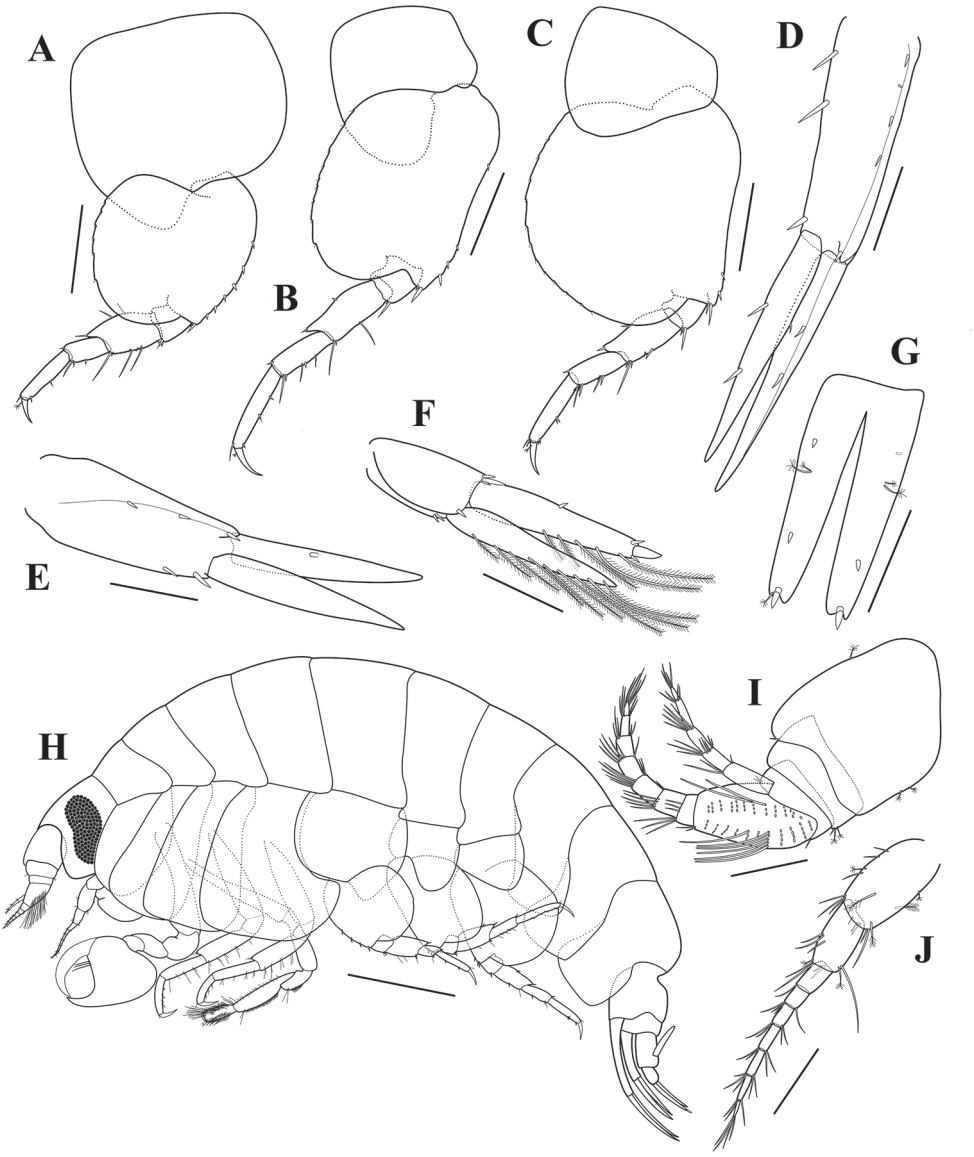


**Figure 7.** *Opisa takafuminakanoi* Narahara-Nakano, Kakui & Tomikawa, 2016, adult male, NIBRIV0000880624, 8.8 mm **A** pereopod 5 **B** pereopod 6 **C** pereopod 7 **D** uropod 1 **E** uropod 2 **F** uropod 3 **G** telson. Adult female, NIBRIV0000880625, 8.7 mm **H** habitus **I** antenna 1 **J** antenna 2. Scale bars: 0.4 mm (**A–C**), 0.2 mm (**D–G, I–J**), 1.0 mm (**H**).

Telson (Fig. 7G) elongated, length 2.25× width, cleft 87% of its length, dorsolaterally each lobe with 2 small robust setae and 2 unequal penicillate setae, apically with 1 stout seta and 1 penicillate seta.

*Adult female*: body (Figs 5B, 7H) about 8.7 mm long. Head similar to that of male except more rounded lateral cephalic lobe.

Antenna 1 (Fig. 7I) stout, similar to that of male except peduncular article 1 with 1 penicillate seta dorsally and 2 penicillate setae ventrally; flagellum 8-articulate, calceoli absent; accessory flagellum 5-articulate, article 1 slightly elongated.

Antenna 2 (Fig. 7J) slender, much shorter than that of male, peduncular articles 4–5 shorter than those of male; flagellum 7-articulate, calceoli absent.

**Remarks.** *Opisa takafuminakanoi* Narahara-Nakano, Kakui & Tomikawa, 2016 is similar to *O. eschrichtii* (Krøyer, 1842) in terms of the following characteristics: 1) epimeron 3 round and smooth posteriorly; 2) gnathopod 1 enlarged, with strongly arched dactylus; 3) gnathopod 1, without "palisade" palmar robust setae; 4) coxa 5 longer than length of basis; and 5) uropod 3, rami with plumose setae. However, *O. takafuminakanoi* is distinguished from *O. eschrichtii* by a vestigial lacinia mobilis on the left mandible, the developed posterior lobe of coxa 5, and the unarmed inner ramus of uropod 2. Our specimens are consistent with the original description provided by Narahara-Nakano et al. (2016).

**Distribution.** Japan, South Korea (East Sea).

### Key to the species of genus *Opisa*

Modified from Narahara-Nakano et al. 2016.

| | | |
|---|---|---|
| 1 | Epimeron 3, posterior margin smooth; maxilliped, outer plate not reaching distal margin of palp article 3 | **2** |
| – | Epimeron 3, posterior margin crenulated or denticulated; maxilliped, outer plate almost reaching distal margin of palp article 3 | ***O. tridentata*** |
| 2 | Gnathopod 1, chela small, dactylus nearly straight, palm of propodus straight, lined with a row of robust setae | **3** |
| – | Gnathopod 1, chela large, dactylus strongly curved, palm of propodus concave, without a row of robust setae | **4** |
| 3 | Gnathopod 1, palm of propodus lined with close-set "palisade" robust setae; uropod 3, rami without marginal setae | ***O. odontochela*** |
| – | Gnathopod 1, palm of propodus lined with blunt robust setae; uropod 3, rami with marginal setae | ***O. parvimana* sp. nov.** |
| 4 | Left mandible, lacinia mobilis developed; coxa 5, posterior lobe weakly developed; uropod 2, inner ramus with robust setae | ***O. eschrichtii*** |
| – | Left mandible, lacinia mobilis vestigial; coxa 5, posterior lobe well developed; uropod 2, inner ramus without robust setae | ***O. takafuminakanoi*** |

## Acknowledgements

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# A new species of *Princaxelia* from Shinkai Seep Field, Mariana Trench (Crustacea, Amphipoda, Pardaliscidae)

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## Abstract

A new pardaliscid amphipod, *Princaxelia marianaensis* **sp. nov.**, is described from a single female captured at the Shinkai Seep Field, Mariana Trench, from a depth of 5,689–5,683 m. A key to species of *Princaxelia* is provided. This is the first species of *Princaxelia* to be described from the Mariana Trench, and the second report of this genus from this region.

## Keywords

COI, deep sea, first record, hadal zone, *Princaxelia marianaensis*, systematics

## Introduction

The benthic amphipod genus *Princaxelia* Dahl, 1959 occurs in deep waters of the Pacific Ocean (Lörz 2010). To date, four species have been described: *P. abyssalis* Dahl, 1959 from 6,435–9,530 m in the Aleutian, Kurile-Kamchatka, Izu-Ogasawara, Yap, Japan, Philippine, Bougainville, and Kermadec Trenches (Kamenskaya 1981, 1997); *P. jamiesoni* Lörz, 2010 from 7,055–9,583 m in the Kurile-Kamchatka, Japan, and Izu-Ogasawara Trenches (Lörz 2010; Jażdżewska and Mamos 2019); *P. magna* Kamenskaya, 1977 from 7,190–7,250 m in the Yap Trench; and *P. stephenseni* Dahl, 1959, the type species of the genus, from 1,505 m off the coast of Iceland. *Princaxelia abyssalis* and *P. jamiesoni* are reported to prey on other amphipods, suggesting that this genus is carnivorous (Jamieson et al. 2012).

The Shinkai Seep Field is a serpentinized, peridotite-hosted, cold-seep system which hosts an aggregation of chemosynthesis-based communities including *Abyssogena* clam, *Provanna* gastropod, and *Phyllochaetopterus* polychaete species. It is located northeast of the Challenger Deep, the deepest part of the Mariana Trench (Ohara et al. 2012; Okutani et al. 2013, 2016; Chen et al. 2018; Watanabe et al. in press). During one submersible dive on an expedition to this seep by R/V *Yokosuka*, a single specimen of a species referable to *Princaxelia* was collected. This is the first record of an identified *Princaxelia* species from the Mariana Trench. We here describe and illustrate this species as new.

## Material and methods

### Samples

The single *Princaxelia* specimen was collected from the Mariana Trench during dive 1402 of the deep-submergence vehicle (DSV) *Skinkai 6500* aboard R/V *Yokosuka* (cruise YK14-13, PI: Yasuhiko Ohara) by H. K. Watanabe (Fig. 1). Aboard the ship, the specimen was fixed and preserved in 99.5% ethanol. The specimen was sorted by K. Tanaka in the laboratory.

The holotype of *P. jamiesoni*, which was collected from the Japan Trench, was borrowed from the Tsukuba Collection Center of the National Museum of Nature and Science, Tokyo (NSMT-Cr 21250, female BL 56.2 mm), for comparison.

### Morphology

Appendages were dissected in 99% ethanol and mounted using gum chloral medium on glass slides with the aid of a stereomicroscope (Olympus SZX7). Appendages were examined by light microscopy (Nikon Eclipse Ni) and illustrated using a camera lucida. Body length (BL), from the tip of the rostrum to the base of the telson along the dorsal curvature, was measured to the nearest 0.1 mm. The only known specimen, the holotype, has been deposited in the collections of the American Museum of Natural History (**AMNH**).

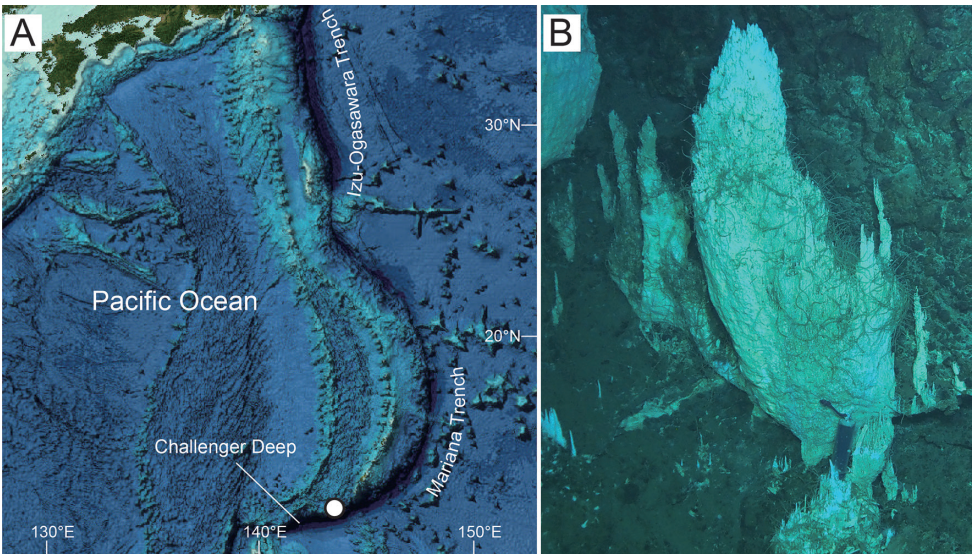


**Figure 1.** Sampling location and habitat of *Princaxelia marianaensis* Tomikawa & Watanabe, sp. nov. **A** map indicating sampling location (circle) (map data from GEBCO Compilation Group [2020]) **B** sampling site at 5,686 m depth (Okumura et al. 2016).

### PCR and DNA sequencing

Genomic DNA was extracted from pereopod muscle of the holotype following procedures detailed in Tomikawa et al. (2014). The primer set for the cytochrome c oxidase subunit I (COI) gene (LCO1490 and HCO2198; Folmer et al. 1994) was used for the polymerase chain reaction (PCR) and cycle sequencing reactions. PCR and sequencing followed the methods detailed by Tomikawa et al. (2017). The DNA sequence has been deposited with the International Nucleotide Sequence Database Collaboration (INSDC) through the DNA Data Bank of Japan (DDBJ).

## Systematics

**Family Pardaliscidae Boeck, 1871**
**Genus *Princaxelia* Dahl, 1959**

***Princaxelia marianaensis* Tomikawa & Watanabe, sp. nov.**
http://zoobank.org/B127A8B4-7BDA-4027-A7DA-8C04F61EA6BA
Figures 2–5

**Material examined. *Holotype*:** female (BL 23.9 mm), **AMNH**_IZC 00361360, the surface of the chimney which was named as "Chim 4" in CH 3 site in the Shinkai Seep Field (Okumura et al. 2016), Mariana Trench (11°39.36'N, 143°2.88'W), 5,689–5,683 m, collected by H. K. Watanabe, 17 July 2014.

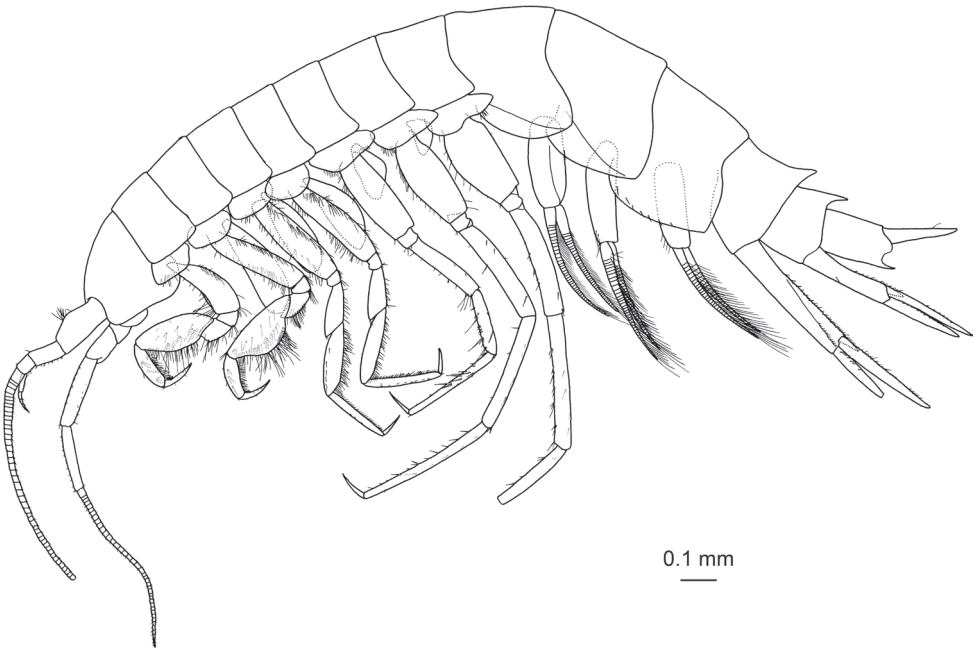


**Figure 2.** *Princaxelia marianaensis* Tomikawa & Watanabe, sp. nov., holotype female (BL 23.9 mm). Habitus, lateral view.

**Diagnosis.** Posterodistal corner of epimeral plate 3 quadrate. Primary flagellum article 1 of female antenna 1 not elongate; accessory flagellum article 1 longer than each of the articles 2–6. Maxilla 1 inner plate with 1 terminal plumose seta; palp article 2 expanded, with 8 or 9 apical robust setae. Dactylus of gnathopods 1 and 2 with three strong projections on posterior margin proximal to base. Dorsal margin of coxa 5 highest at proximal end. Venral margin of coxa 7 weakly concave. Telson lobe uniformly tapering distally.

**Description (female).** ***Head*** (Fig. 2) as long as pereonites 1 and 2 combined; rostrum short, pointed; lateral cephalic corner rounded; eyes absent. Pleon (Fig. 2) with dorsal surfaces of pleonites 1–3 smooth; epimeral plates 1–3 (Fig. 3A–C) with setae on ventral submargin and posterior margin; posterodistal corner of epimeral plates 2 and 3 quadrate. Dorsal margin of urosomites 1 and 2 (Fig. 2) with distally oriented projection.

***Antenna 1*** (Fig. 3D) length 0.3 times BL (distal part broken off); peduncular articles 1–3 with length ratio 1.0 : 0.7 : 0.3; peduncular article 1 broadened, with anterolateral cluster of setae, some weakly plumose; posterior margin of peduncular articles 2 and 3 with clusters of short setae; primary flagellum article 1 length 1.2 times width, 3.0 times as long as article 2; accessory flagellum 6-articulated, article 1 0.9 times as long as articles 2–6 combined; primary flagellum with at least 47 articles.

***Antenna 2*** (Fig. 3E) length 0.4 times BL; anterior margin of peduncular article 2 with setae; peduncular articles 4 and 5 with clusters of short setae on anterior margin, article 4 1.1 times longer than article 5; flagellum with 42 articles.

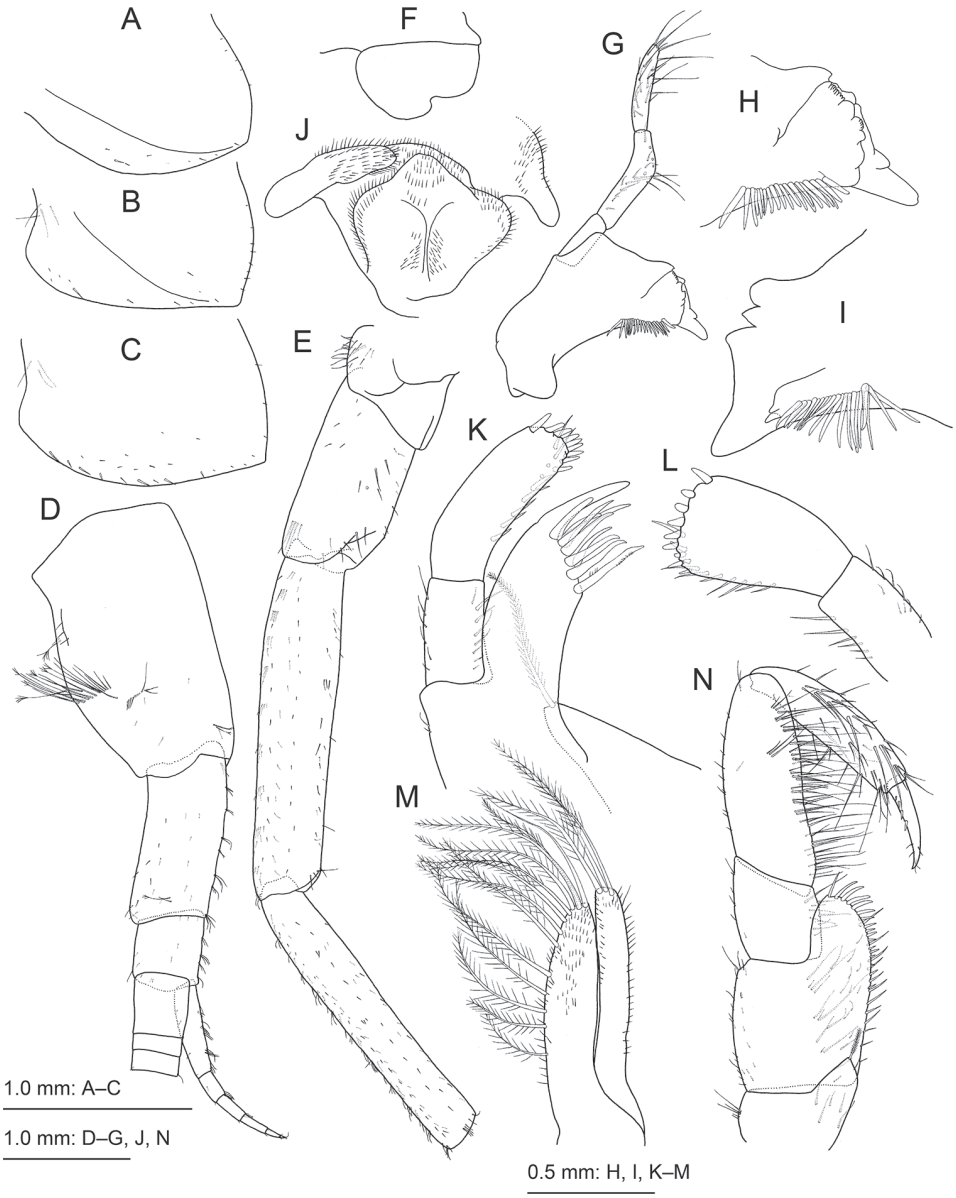


**Figure 3.** *Princaxelia marianaensis* Tomikawa & Watanabe, sp. nov., holotype female (BL 23.9 mm) **A** epimeral plate 1, lateral view **B** epimeral plate 2, lateral view **C** epimeral plate 3, lateral view **D** antenna 1, lateral view, some distal articles of primary flagellum omitted **E** antenna 2, lateral view, flagellum omitted **F** upper lip, anterior view **G** left mandible, medial view **H** left mandible, medial view **I** right mandible, medial view **J** lower lip, anterior view **K** maxilla 1, dorsal view **L** palp of maxilla 1, dorsal view **M** maxilla 2, dorsal view **N** maxilliped, dorsal view.

***Upper lip*** (Fig. 3F) asetose, with asymmetrically incised ventral margin. Mandibles (Fig. 3G–I) slightly asymmetric, incisor margins broad, anteroventral corner with strong tooth; left lacinia mobilis (Fig. 3H) broad, about 0.7 times as long as incisor,

multi-dentate; right incisor (Fig. 3I) with three teeth on proximal to anterodorsal corner; right lacinia weak, with two teeth; accessory setal row of left and right mandibles each with about 20 robust setae; molar absent; mandibular palp 3-articulated with length ratio 1.0 : 1.7 : 1.5; article 1 asetose; article 2 posteriorly reflected, articles 2 and 3 with 18 and 22 setae, respectively. Lower lip (Fig. 3J) with broad outer and distinct inner lobes. Maxilla 1 (Fig. 3K, L) with inner and outer plates and palp; inner plate small with apical plumose seta; outer plate subrectangular, with 9 robust apical setae and strong projection; palp 2-articulate; article 1 with marginal setae; article 2 expanded distally with nine and eight robust setae on apical margin of left and right maxilla 1, respectively, and with apical submargin and medial margin lined with setae. Maxilla 2 (Fig. 3M) with inner plate bearing row of 13 plumose setae along apical to medial margin; outer plate slightly longer than inner plate, with three apical plumose setae. Maxilliped (Fig. 3N) with inner and outer plates and palp; inner plate small, subtriangular, not reaching base of palp, with plumose apical seta and short subapical seta; outer plate oval, reaching base of article 2 of palp, with setae along apical to medial margin; palp 4-articulate, long: article 2 longest with inner marginal rows of setae, article 3 with clusters of setae on dorsal and ventral faces and medial marginal setae, and article 4 slender, with robust setae on medial margin.

***Gnathopod 1*** (Fig. 4A, B) coxa subrectangular, length 1.8 times width, ventral margin straight, posterior submargin and medial face with setae; basis arched, with anterior and posterior margins with numerous setae in a row; posterior margin of merus with sparse setae; carpus oval, length 2.5 times width, posterior margin and medial face setose; propodus slender, length 0.6 times that of carpus, posterior margin weakly convex with setae; dactylus slender, slightly curved, posterior margin with three strong projections proximal to base. Gnathopod 2 (Fig. 4C, D) coxa tapering anteriorly, length 1.8 times width, posterior submargin with setae; basis slender and straight, anterior and posterior margins densely setose; carpus widely produced posteriorly with numerous long setae, length 2.3 times width; propodus and dactylus similar to gnathopod 1.

***Pereopod 3*** (Fig. 4E, F) coxa weakly rounded ventrally, with submarginal setae; basis long, posterior margin strongly setose; merus, carpus, propodus, and dactylus in length ratio 1.0 : 1.4 : 1.4 : 0.5; posterior margin of propodus lined with short setae. Pereopod 4 (Fig. 4G) similar to pereopod 3, with coxa tapering anteriorly. Pereopod 5 (Fig. 4H, I) coxa subtriangular, dorsal margin highest at proximal end, anterior and ventral submargins with setae; basis length 2.9 times width, with clusters of setae on anterior margin proximal to base, posterodistal corner weakly produced; merus, carpus, propodus, and dactylus in length ratio 1.0 : 0.8 : 1.2: 0.3; carpus and propodus with robust setae on anterior and posterior margins. Pereopod 6 (Fig. 4J) coxa weakly concave; basis length 2.5 times width, posterodistal corner quadrate; merus, carpus, propodus, and dactylus in length ratio 1.0 : 1.0 : 1.2: 0.3. Pereopod 7 (Fig. 4K) coxa weakly concave; basis length 1.9 times width, weakly expanded anteriorly, posterodistal corner quadrate.

***Coxal gills*** (Fig. 2) on gnathopod 2, pereopods 3–6; coxal gills 2–4 elongate, coxal gill 2 longest, its length exceeding the distal part of basis of gnathopod 2, coxal gill 6 shortest.

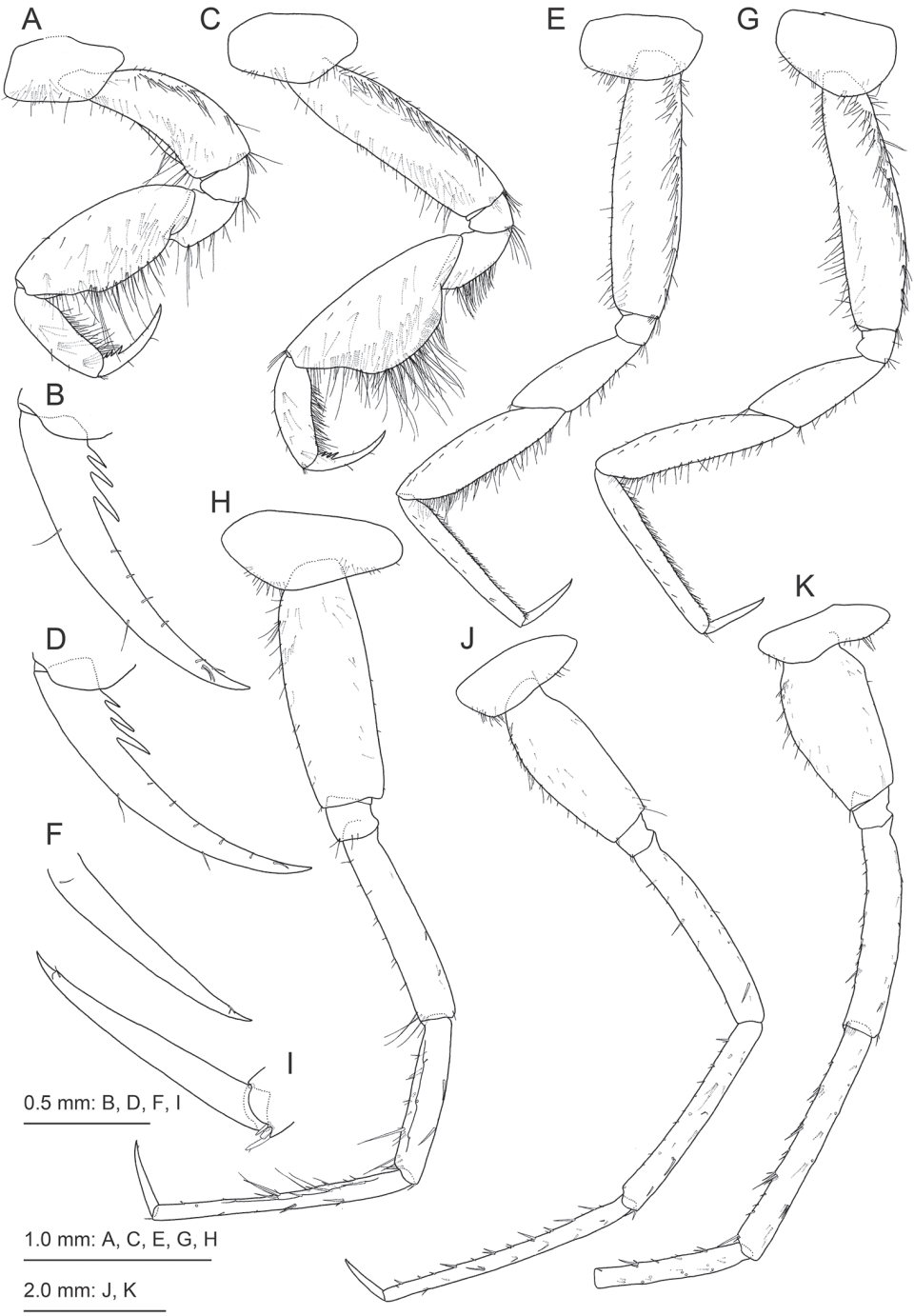


**Figure 4.** *Princaxelia marianaensis* Tomikawa & Watanabe, sp. nov., holotype female (BL 23.9 mm) **A** gnathopod 1, lateral view **B** dactylus of gnathopod 1, lateral view **C** gnathopod 2, lateral view **D** dactylus of gnathopod 2, lateral view **E** pereopod 3, lateral view **F** dactylus of pereopod 3, lateral view **G** pereopod 4, lateral view **H** pereopod 5, lateral view **I** dactylus of pereopod 5 **J** pereopod 6, lateral view **K** pereopod 7, lateral view.

***Pleopods 1–3*** (Fig. 5A–C) each with paired retinacula (Fig. 5B) on inner distal margin of peduncle, and bifid (clothespin) setae (Fig. 5C) on inner basal margin of inner ramus; rami articles wide and flattened.

***Uropod 1*** (Fig. 5D) peduncle longer than rami, with 14 basofacial setae, distomedial peduncular projection very strong; inner ramus length 0.8 times that of peduncle, outer ramus distally damaged, rami with setal row along medial and lateral margins. Uropod 2 (Fig. 5E) peduncle slightly longer than rami, with four basofacial setae, distomedial peduncular spine shorter than that of uropod 1; inner ramus length 1.2 times that of outer ramus, rami with setal row along medial and lateral margins. Uropod 3 missing (damaged).

***Telson*** (Fig. 5F) length 2.3 times width, with cleft extending 80% its length; lobes tapering distally with facial setae; apex of each lobe shallowly incised with small robust seta.

**Etymology.** The specific name is an adjective derived from the type locality, the Mariana Trench.

**DNA sequence.** A single nucleotide sequence of COI was obtained from the holotype (AMNH_IZC 00361360; 658 bp).

**Remarks.** The morphologies of *P. marianaensis* sp. nov. and congeners are summarized in Table 1. *Princaxelia marianaensis* sp. nov. is most similar to *P. abyssalis* Dahl, 1959 in having a short first flagellar article of the female antenna 1, a weakly setose maxilla 1, coxa 5 with its dorsal margin highest at the proximal end and its distal margin rounded, and a uniformly tapering telson. However, *P. marianaensis* sp. nov. differs from the description of *P. abyssalis* in having the posterodistal corner of epimeral plate 3 quadrate in *P. marianaensis* sp. nov. but rounded in *P. abyssalis*; the

**Table 1.** Morphological comparison of *Princaxelia* species.

| | ***P. marianaensis* Tomikawa & Watanabe, sp. nov.** | ***P. abyssalis* Dahl, 1959** | ***P. jamiesoni* Lörz, 2010** | ***P. magna* Kamenskaya, 1977** | ***P. stephenseni* Dahl, 1959** |
|---|---|---|---|---|---|
| Maximum body size | female 23.9 mm | male 21 mm, female 32 mm | male 57 mm, female 61 mm | male 52 mm | male 10 mm, female 11 mm |
| Epimeral plate 3 posterodistal corner | quadrate | rounded | quadrate | quadrate | weakly rounded |
| Dorsal projections on urosomites 1 and 2 | pointing toward distal end | unknown | pointing toward distal end | pointing upright | pointing toward distal end |
| Upper lip | strongly asymmetrical | unknown | slightly asymmetrical | strongly asymmetrical | nearly asymmetrical |
| Maxilla 1 palp article 2 | expanded | expanded | expanded | expanded | not expanded |
| Maxilla 1 palp article 2 | 9 apical robust setae | less than 14 apical robust setae | 25 apical robust setae | approx. 10 apical robust setae | 7 apical robust setae |
| Maxilla 1 inner plate | 1 plumose seta | 1 plumose seta | 1 plumose seta | 6 plumose setae | 1 plumose seta |
| Female antenna 1 primary flagellum article 1 | not elongated | not elongated | elongated | unknown | elongated |
| Female antenna 1 accessory flagellum article 1 | longer than each of the rest | equal to length of remaining articles | longer than each of the rest | unknown | unknown |
| Gnathopods 1 and 2 dactyli | 3 strong projections near the base | unknown | 8–9 strong projections near the base | 4 strong projections near the base | unknown (absent?) |
| Coxa 5 dorsal margin | highest at proximal end | highest at proximal end | straight | convex | straight / convex |
| Coxa 5 distal margin | rounded | rounded | rounded | slightly pointed | straight |
| Coxa 7 ventral margin | shallowly concave | straight | slightly concave | slightly concave | straight |
| Telson lobe | uniformly tapering distally | uniformly tapering distally | tapering from distal 1/3 | weakly tapering distally | unknown |
| References | This study | Dahl (1959) | Lörz (2010); this study | Kamenskaya (1977) | Dahl (1959); Lörz (2010) |

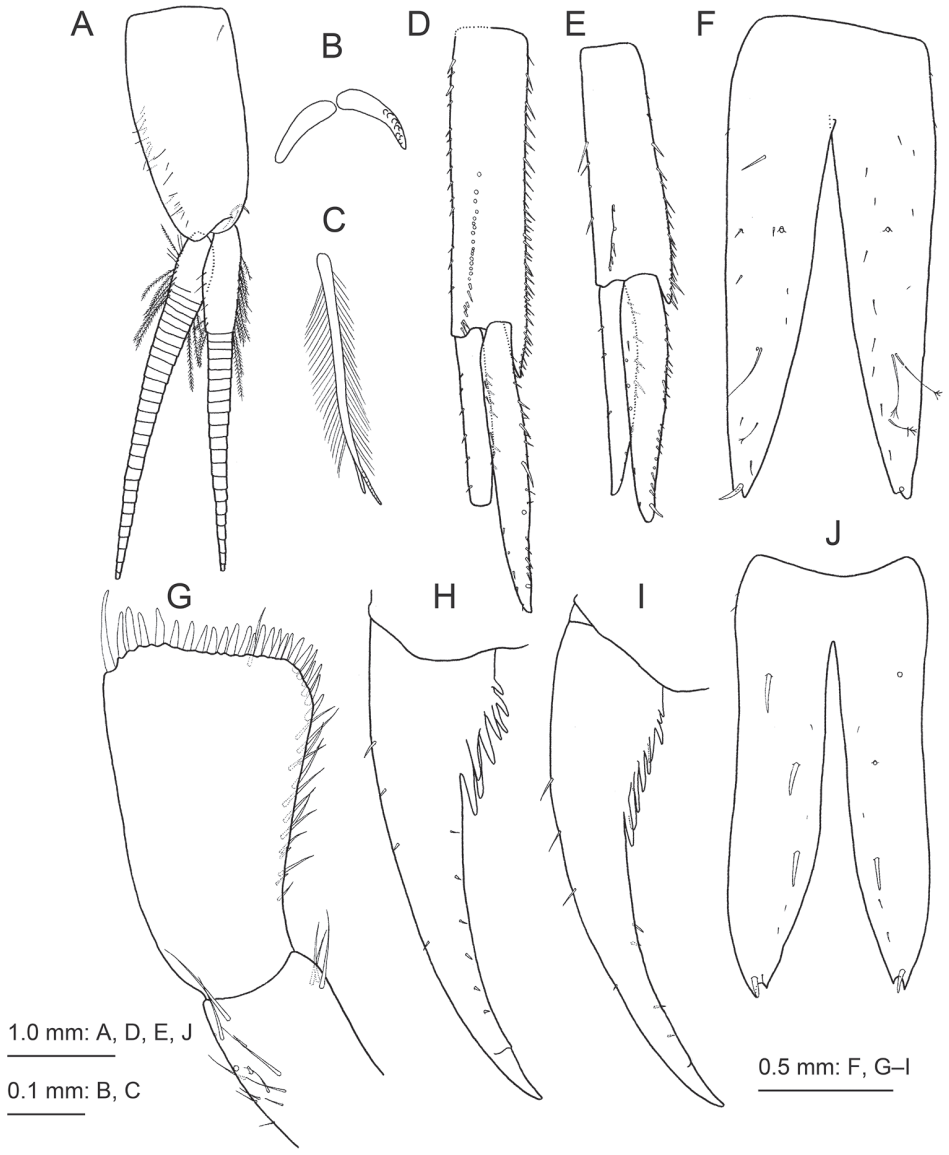


**Figure 5.** *Princaxelia marianaensis* Tomikawa & Watanabe, sp. nov., holotype female (BL 23.9 mm) **A** pleopod 1, anterior view, some setae on rami omitted **B** retinacula on peduncle of pleopod 1, anterior view **C** bifid (clothespin) plumose seta on inner basal margin of inner ramus of pleopod 1, anterior view **D** uropod 1, dorsal view, distal part of outer ramus broken **E** uropod 2, dorsal view **F** telson, dorsal view. *Princaxelia jamiesoni* Lörz, 2010, holotype female (BL 56.2 mm) **G** palp of maxilla 1, dorsal view **H** dactylus of gnathopod 1, lateral view **I** dactylus of gnathopod 2, medial view **J** telson, dorsal view.

accessory flagellum article 1 of the female antenna 1 longer than each of the articles 2–6 in *P. marianaensis* sp. nov. but equal to the length of the remaining segments in *P. abyssalis*; and the ventral margin of the coxa 7 weakly concave in *P. marianaensis* sp. nov. but straight in *P. abyssalis*.

*Princaxelia jamiesoni* Lörz, 2010 was described from 7,703 m and 9,316 m in the Japan and Izu-Ogasawara trenches, respectively (Lörz 2010), and subsequently from 7,055–9,583 m in the Kurile-Kamchatka Trench (Jażdżewska and Mamos 2019). Examination of the holotype of *P. jamiesoni* reveals new features not originally described which facilitate differentiation of this species from *P. marianaensis* sp. nov.: the palp article 2 of the maxilla 1 bears eight or nine robust apical setae in *P. marianaensis* sp. nov. but 25 robust apical setae in *P. jamiesoni* (Fig. 5G); the dactylus of gnathopods 1 and 2 has three strong projections proximal to its base in *P. marianaensis* sp. nov., but eight or nine strong projections proximal to the base of the dactylus in *P. jamiesoni* (Fig. 5H, I); and the telson lobe uniformly tapers distally in *P. marianaensis* sp. nov. but tapers from the distal 1/3 in *P. jamiesoni* (Fig. 5J). While two projections on the dactylus of the left gnathopod 2 were originally described for *P. jamiesoni*, we report nine projections on the right gnathopod 2 of the holotype; we believe that Lörz (2010) described the damaged left gnathopod 2.

The morphology of *Princaxelia* is consistent with an animal that swims in that its body is streamlined, flat, and has well-developed pleopods (Lörz 2010). Analyses of the locomotion of *Princaxelia* species demonstrate they have a high swimming ability – a trait useful for preying on other amphipods in hadal trenches (Jamieson et al. 2012). Amphipods lack a planktonic larval stage and generally have low dispersal ability (Chapman 2007). Judging from known habitat depths of *Princaxelia*, with the exception of the bathypelagic *P. stephenseni*, the distributions of species might be expected to be restricted to individual trenches. However, *P. abyssalis*, and especially

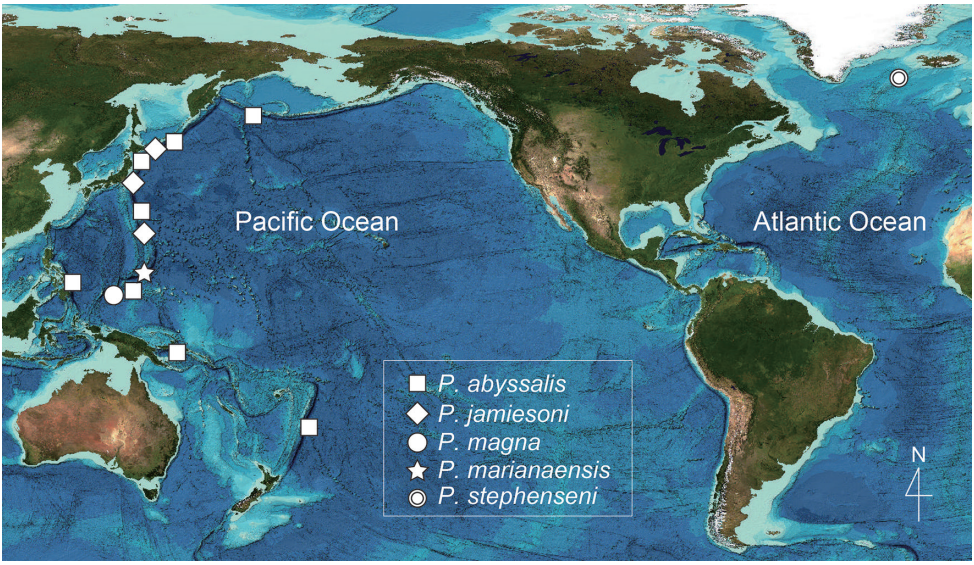


**Figure 6.** Geographical distributions of the species of *Princaxelia* (map data from GEBCO Compilation Group [2020]). The exact location of the distribution of *P. abyssalis* in the Aleutian Trench is uncertain.

*P. jamiesoni*, are reported from multiple trenches (Fig. 6) (Kamenskaya 1981, 1997; Lörz 2010; Jażdżewska and Mamos 2019). Deep-sea amphipod species previously regarded as widely distributed have since been found to contain cryptic species (e.g., Narahara-Nakano et al. 2018). Lörz (2010) also considered that *P. abyssalis*, as reported from multiple trenches by Kamenskaya (1981), may contain other or undescribed species. It is possible that *P. abyssalis* and *P. jamiesoni* represent species complexes, but a greater understanding of species diversity of this hadal-dwelling genus will require additional genetic and morphological analyses.

### Key to species of *Princaxelia* modified from Lörz (2010)

We added *P. marianaensis* sp. nov. to the key by Lörz (2010) and modified the key to include the characteristics of the telson, which was not considered by Lörz (2010).

1 Palp article 2 of maxilla 1 expanded ..... **2**
– Palp article 2 of maxilla 1 not expanded ..... ***P. stephenseni*** **Dahl, 1959**
2 Inner plate of maxilla 1 with 1 terminal plumose seta ..... **3**
– Inner plate of maxilla 1 with several plumose setae ..... ***P. magna*** **Kamenskaya, 1977**
3 Primary flagellum article 1 of female antenna 1 not elongate; dorsal margin of coxa 5 highest at proximal end; telson lobe uniformly tapering distally ... **4**
– Primary flagellum article 1 of female antenna 1 elongate; dorsal margin of coxa 5 straight; telson lobe tapering from distal 1/3 ..... ***P. jamiesoni*** **Lörz, 2010**
4 Posterodistal corner of epimeral plate 3 rounded; accessory flagellum article 1 of female antenna 1 equal to length of remaining articles; ventral margin of coxa 7 straight ..... ***P. abyssalis*** **Dahl, 1959**
– Posterodistal corner of epimeral plate 3 quadrate; accessory flagellum article 1 of female antenna 1 longer than each of remaining articles; ventral margin of coxa 7 weakly concave ..... ***P. marianaensis*** **Tomikawa & Watanabe, sp. nov.**

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We thank the captain and crew of the R/V *Yokosuka* and the operation team of DSV *Shinkai 6500* for their cooperation in the YK14-13 cruise led by Y.O. The Shinkai Seep Field is a part of the Mariana Trench Marine National Monument of the United States of America, and we greatly appreciate the U.S. Fish and Wildlife Service for approving our study in the monument under the special use permit #12541-14001. The sample number under this permit used in the present study is #12541-14001-6K-1402-B01. This work was partly supported by the Japan Society for the Promotion of Science KAKENHI grants JP17K15174 and JP17H00820 to the first author.

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# Two new species of *Coniopteryx* Curtis from China (Neuroptera, Coniopterygidae)

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## Abstract

Two new species of Coniopterygidae, *Coniopteryx* (*Coniopteryx*) *tenuisetosa* **sp. nov.**, and *Coniopteryx* (*Coniopteryx*) *serrata* **sp. nov.**, are described from China. Both species differ from congeners in characters of the male genitalia. *Coniopteryx* (*Coniopteryx*) *alticola* Sziráki, 2002, is recorded from China for the first time. A key to species of the genus *Coniopteryx* from China is presented.

## Keywords

Dustywings, faunistics, identification key, lacewings, morphology, taxonomy

## Introduction

Coniopterygidae, or dustywings – after the wax covering their bodies – are one of the most diverse lineages of Neuroptera, including 571 known species (Oswald and Machado 2018). Coniopterygids are common and often abundant in woody environments worldwide, though they are easily overlooked due to their small size, being the dwarfs among lacewings. Nevertheless, dustywings are of major phylogenetic interest, as they are the sister group to all the other Neuroptera, diverging from them in the Permian (Winterton et al. 2018; Vasilikopoulos et al. 2020). Their evolutionary history has been characterized by miniaturization, with a reduction of their overall body

size, with major impacts on their morphology and anatomy (Randolf et al. 2017; Randolf and Zimmermann 2019). Like most lacewings, dustywings are predators both as larvae and adults, feeding on small arthropods such as mites, scale insects and aphids (Pantaleoni 2007). Coniopterygidae are divided in three subfamilies, Brucheiserinae, Aleuropteryginae and Coniopteryginae, of which the last group is the richest in species (Oswald and Machado 2018; Handschuh and Aspöck 2020). The genus *Coniopteryx* Curtis (1834) is in turn the most diverse group of Coniopteryginae attaining a sub-cosmopolitan distribution (Meinander 1972; Sziráki 2011). Meinander (1972) divided this genus into six subgenera based on morphology of genitalia: *Coniopteryx* s. str. (77 spp.), *Xeroconiopteryx* Meinander, 1972 (85 spp.), *Protoconiopteryx* Meinander, 1972 (1 sp.) *Scotoconiopteryx* Meinander, 1972 (33 spp.), *Holoconiopteryx* Meinander, 1972 (8 spp.), and *Metaconiopteryx* Meinander, 1972 (4 spp.). Eleven additional species are not presently allocated to a subgenus (see also Meinander 1990; Sziráki 2011, 2015, Martins and Amorim 2016). Twenty-six species of *Coniopteryx* are known for China, belonging to the subgenera *Coniopteryx* (22 spp.) and *Xeroconiopteryx* (4 spp.). This paper describes two new species of *Coniopteryx* s. str. from China. We also report for the first time the presence of *Coniopteryx alticola* Sziráki 2002 in China, increasing the number of *Coniopteryx* species known from this country to 29.

## Material and methods

Examined specimens are deposited in the Entomological Museum of China Agricultural University, Beijing (**CAU**), which are preserved in 95% ethyl alcohol. The abdomen was dissected from the body and macerated in a heated solution of 5% KOH for 5 minutes, then rinsed in water and 95% ethyl ethanol. And finally, the cleared abdomen was transferred to glycerol for dissection and study. After examination, the abdomen was preserved in glycerol and stored in a microtube. The head and the thorax of the specimen were preserved in 95% ethyl alcohol and stored in another microtube. Morphological terminology mostly follows Meinander (1972), Aspöck and Aspöck (2008) and Handschuh and Aspöck (2020). Specimens were examined with an Optec SZ760 stereomicroscope. Photos were taken with a Nikon D5300 digital camera attached to a Leica DM2500 stereomicroscope. The resulting images were edited and processed with Adobe Photoshop CC 2018.

## Taxonomy

**Family Coniopterygidae Burmeister, 1839**
**Subfamily Coniopteryginae Burmeister, 1839**
**Genus *Coniopteryx* Curtis, 1834**

**Subgenus *Coniopteryx* (s. str.) Curtis, 1834**

**Type species.** *Coniopteryx tineiformis* Curtis, 1834.

**Diagnosis.** Male genitalia: gonocoxites 9 and sternite 9 as distinct sclerites; gonocoxites 9 divided into a pair of lateral sclerites; sternite 9 about as broad as high in lateral view, with a prominent lateral process, forming a dorso-caudal angle, median apical incision present; gonapophyses 10 generally sclerotized (Meinander 1972; Sziráki 2011; Handschuh and Aspöck 2020).

## Key to the species of *Coniopteryx* from China (males)

Note: *Coniopteryx* (*Coniopteryx*) *abdominalis* Okamoto, 1905 is not included in the key as the specimen is unavailable for study.

1 Apical part (stylus) arising well before the caudal end of basal part (gonarcus) in gonocoxites 9 (Fig. 1a–c) subgenus *Xeroconiopteryx* ..... **2**
– Apical part (stylus) arising from the caudal end of basal part (gonarcus) in gonocoxites 9 (Figs 1d, 6a, b, 8a, b, 10a, b) subgenus *Coniopteryx* ..... **5**
2 Anterior margin arched on sternite 9 laterally (Fig. 1a) ..... ***C.* (*X.*) *mongolica***
– Anterior margin straight on sternite 9 laterally (Fig. 1b, c) ..... **3**
3 Apodeme along anterior margin ventrally incomplete (Fig. 2a) ..... ***C.* (*X.*) *qiongana***
– Apodeme along anterior margin ventrally complete (Fig. 2b) ..... **4**
4 Apical part (stylus) of gonocoxites 9 slender laterally (Fig. 1b) .... ***C.* (*X.*) *minana***
– Apical part (stylus) of gonocoxites 9 widening in middle part laterally (Fig. 1c) ..... ***C.* (*X.*) *unguigonarcuata***
5 Male head with prominent frontal lobe (Fig. 5a–c) *Coniopteryx lobifrons* group (3 species) ..... **6**
– Male head without prominent frontal lobe (Figs 7b, 9b) ..... **8**
6 Distal part of gonocoxites 10 hammer-like laterally (Fig. 3a) ..... ***C.* (*C.*) *dactylifrons***
– Distal part of gonocoxites 10 not hammer-like laterally (Figs 3b, 6a, b) ..... **9**
7 Gonocoxites 10 subtriangular apically laterally (Fig. 3b) ..... ***C.* (*C.*) *protrufrons***
– Gonocoxites 10 not subtriangular apically laterally (Fig. 6a, b) .... ***C.* (*C.*) *alticola***
8 Male antennae with peculiar outgrowths (Fig. 4a–d) *Coniopteryx falciger* group (4 species) ..... **9**
– Male antennae without peculiar outgrowths (Figs 7a, b, 9a, b) ..... **12**
9 The first two flagellar segments with acute projections (Fig. 4a) ..... ***C.* (*C.*) *bispinalis***
– The first two flagellar segments without acute projections (Fig. 4b–d) ..... **10**
10 The last flagellar segments with a curved claw-like hair (Fig. 4b) ..... ***C.* (*C.*) *prehensilis***
– The last flagellar segments without claw-like hairs (Fig. 4c, d) ..... **11**
11 Antennae with one long bristle on middle segments (Fig. 4c) ..... ***C.* (*C.*) *unispinalis***
– Antennae with two acute projections on middle segments (Fig. 4d) ..... ***C.* (*C.*) *gibberosa***

12 Distal part of gonocoxites 10 pick-like (Fig. 10a, b, g) or hammer-like in shape (Fig. 3c, d) *Coniopteryx tineiformis* group (4 species) ..... **13**
– Distal part of gonocoxites 10 not pick- and hammer-like in shape (Figs 3f–h, 8a, b) *Coniopteryx exigua* group (13 species) ..... **16**
13 Bottom of median incision rounded in a U-shape (Fig. 2c) .... ***C.* (*C.*) *wuyishana***
– Bottom of median incision narrowing in a V-shape (Fig. 10e, f) ..... **14**
14 Processus apicalis of gonocoxites 10 pick-like (Fig. 10a, b) ..... ***C.* (*C.*) *serrata* sp. nov.**
– Processus apicalis of gonocoxites 10 hammer-like (Fig. 3c, d) ..... **15**
15 Median incision deep in ventral view (Fig. 2d) ..... ***C.* (*C.*) *alifera***
– Median incision shallow in ventral view (Fig. 2e) ..... ***C.* (*C.*) *pygmaea***
16 Anterior margin arched on sternite 9 laterally (Fig. 1d) ..... ***C.* (*C.*) *praecisa***
– Anterior margin straight on sternite 9 laterally (Fig. 8a, b) ..... **17**
17 Distal part of gonocoxites 10 sickle-like in shape (Fig. 3e) .... ***C.* (*C.*) *crispicornis***
– Distal part of gonocoxites 10 not sickle-like in shape (Figs 3f–h, 8a, b) ..... **18**
18 Basal flagellar segments more than three times as long as wide (Fig. 4e) ..... ***C.* (*C.*) *miraparameris***
– Basal flagellar segments at most two times as long as wide (Fig. 7a, b) ..... **19**
19 Distal part of gonocoxites 10 widening abruptly (Meinander 1972: 245, fig. 156) ..... ***C.* (*C.*) *pallescens***
– Distal part of gonocoxites 10 not widening abruptly (Figs 3f–h, 8g) ..... **20**
20 Caudal edge of gonocoxites 10 serrate apically (Fig. 8g) ..... ***C.* (*C.*) *tenuisetosa* sp. nov.**
– Caudal edge of gonocoxites 10 not serrate apically (Fig. 3f–h) ..... **21**
21 Distal part of gonocoxites 10 directed downwards perpendicularly (Fig. 3f) ..... ***C.* (*C.*) *aspoecki***
– Distal part of gonocoxites 10 not directed downwards perpendicularly (Fig. 3g, h) ..... **22**
22 Middle part of gonocoxites 10 curved downward in a blunt angle (Fig. 3g) ..... **23**
– Middle part of gonocoxites 10 not curved downward (Fig. 3h) ..... **24**
23 Median incision U-shaped (Meinander 1972: 244, fig. 155) ..... ***C.* (*C.*) *sularis***
– Median incision V-shaped (Fig. 2f) ..... ***C.* (*C.*) *choui***
24 Sternite 9 with strong longitudinal apodeme (Fig. 2g) ..... ***C.* (*C.*) *plagiotropa***
– Sternite 9 without longitudinal apodeme (Fig. 2h–j) ..... **25**
25 Median incision almost equal to the half of width of sternite 9 (Fig. 2h) ..... **26**
– Median incision smaller than the half of width of sternite 9 (Fig. 2i, j) ..... **27**
26 Median incision very deep and narrow (Fig. 2h) ..... ***C.* (*C.*) *compressa***
– Median incision very shallow and wide (Meinander 1972: 238, fig. 151) ..... ***C.* (*C.*) *ambigua***
27 Median incision without a transverse inner plate in caudal view (Fig. 1e) ..... ***C.* (*C.*) *exigua***
– Median incision with a transverse inner plate in caudal view (Fig. 1f) ..... ***C.* (*C.*) *guangxiana***

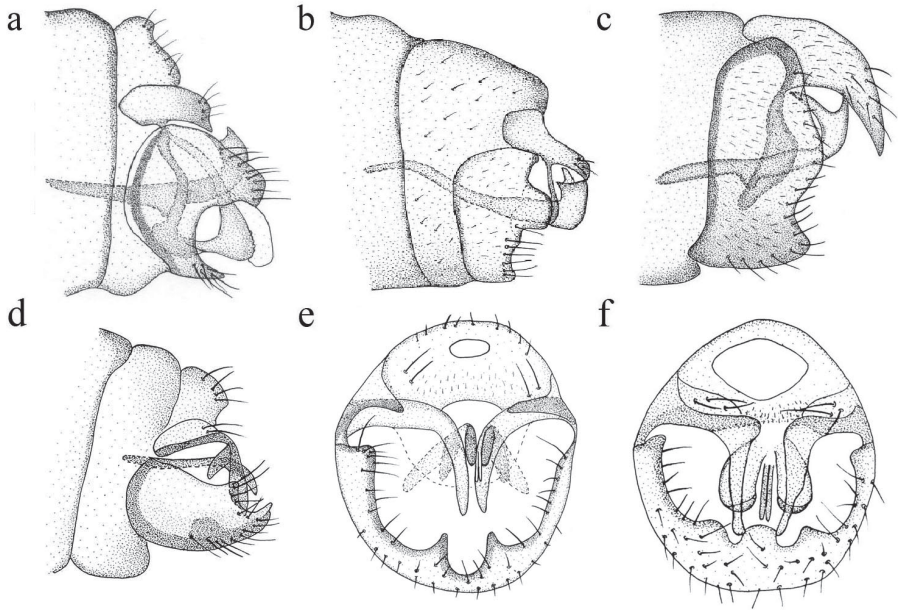


**Figure 1.** Genitalia of *Coniopteryx* species **a** *C. mongolica* (lateral view) **b** *C. minana* (lateral view) **c** *C. unguigonarcuata* (lateral view) **d** *C. praecisa* (lateral view) **e** *C. exigua* (caudal view) **f** *C. guangxiana* (caudal view).

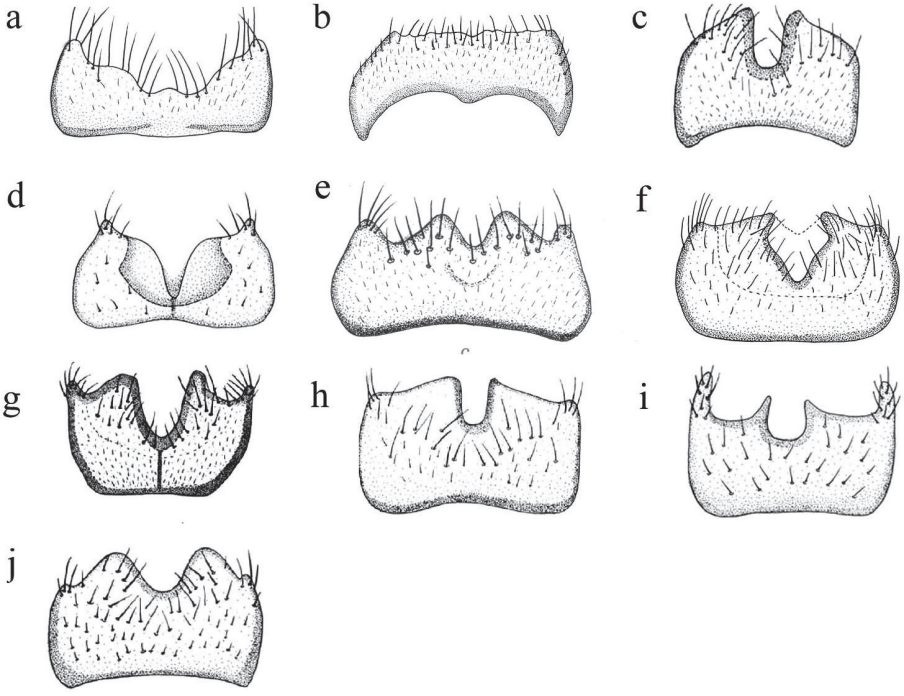


**Figure 2.** Sternite 9 of *Coniopteryx* species, ventral view **a** *C. qiongana* **b** *C. unguigonarcuata* **c** *C. wuyishana* **d** *C. alifera* **e** *C. pygmaea* **f** *C. choui* **g** *C. plagiotropa* **h** *C. compressa* **i** *C. exigua* **j** *C. guangxiana*.

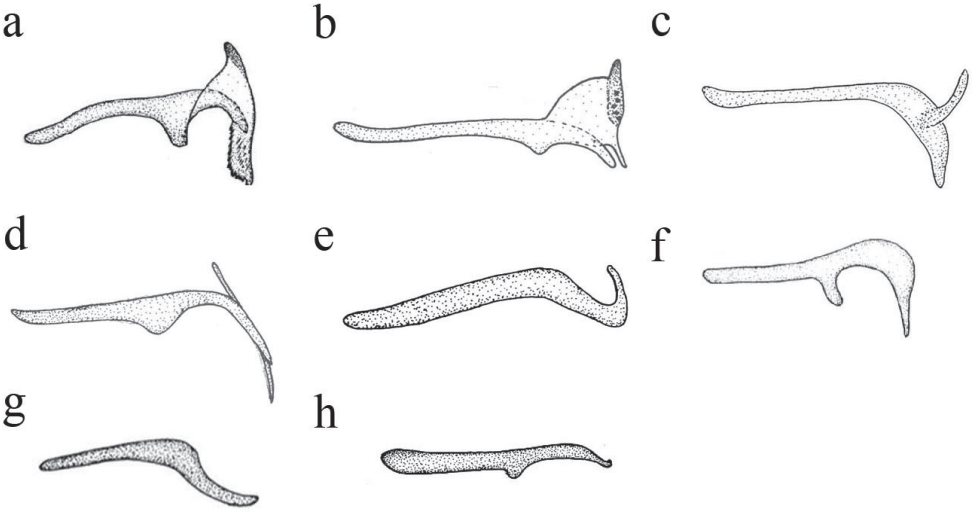


**Figure 3.** Gonocoxites 10 of *Coniopteryx* species, lateral view **a** *C. dactylirons* **b** *C. protrufrons* **c** *C. alifera* **d** *C. pygmaea* **e** *C. crispicornis* **f** *C. aspoecki* **g** *C. choui* **h** *C. plagiotropa*.

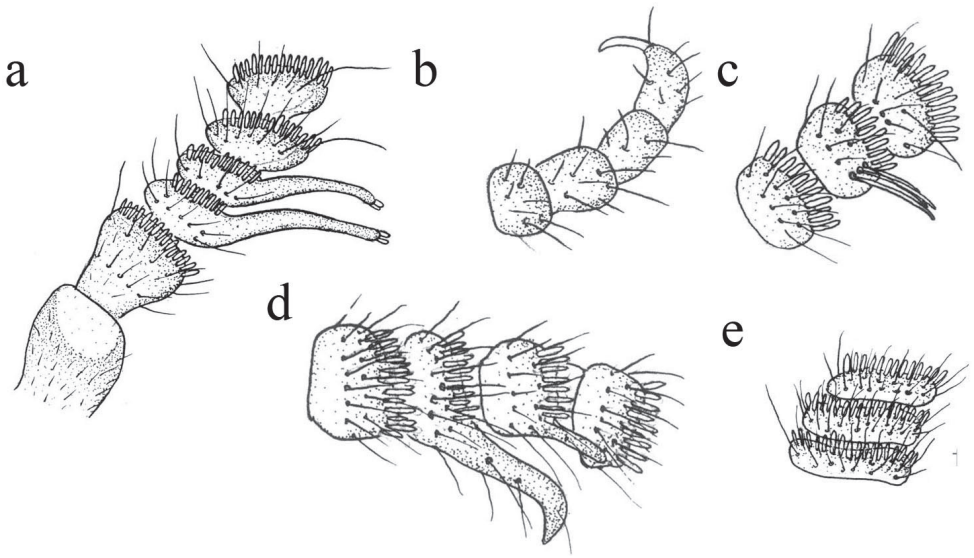


**Figure 4.** Antennae of *Coniopteryx* species **a** *C. bispinalis* (antennal segments 1–6) **b** *C. prehensilis* (distal part of antennal segments) **c** *C. unispinalis* (antennal segments 11–13) **d** *C. gibberosa* (antennal segments 8–11) **e** *C. miraparameris* (antennal segments 8–10).

### *Coniopteryx* (*Coniopteryx*) *alticola* Sziráki, 2002

Figs 5, 6

**Material examined.** 1 male, China: Yunnan (Province): Puer (City): Meizihu Park, [22.7551°N, 100.9845°E], 20.iii.2019, leg. Yaru Zhao. 3 males, China: Yunnan

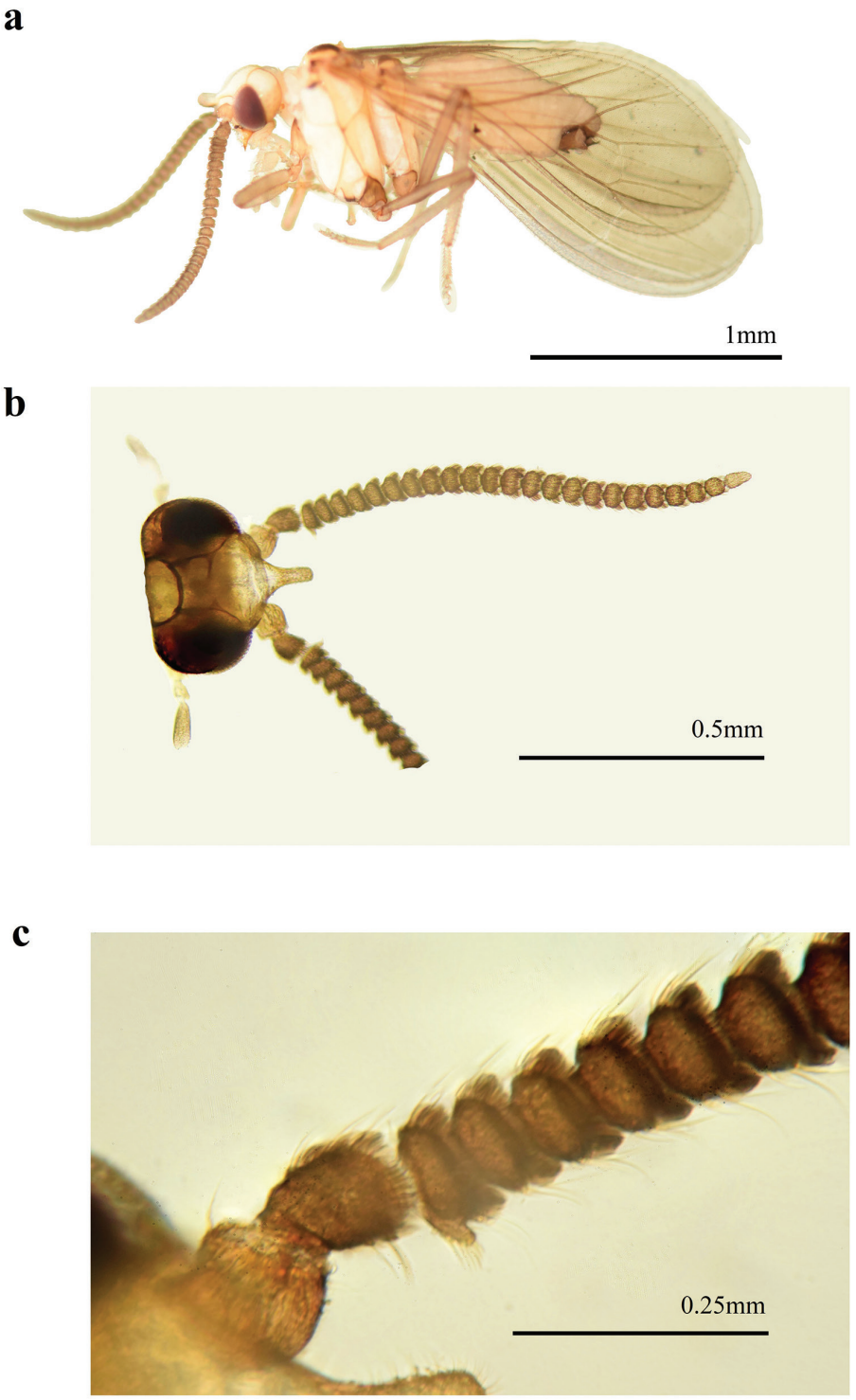


**Figure 5.** *Coniopteryx* (*Coniopteryx*) *alticola* Sziráki, 2002, male **a** habitus, lateral view **b** head, dorsal view **c** male, first flagellomere, dorsal view.

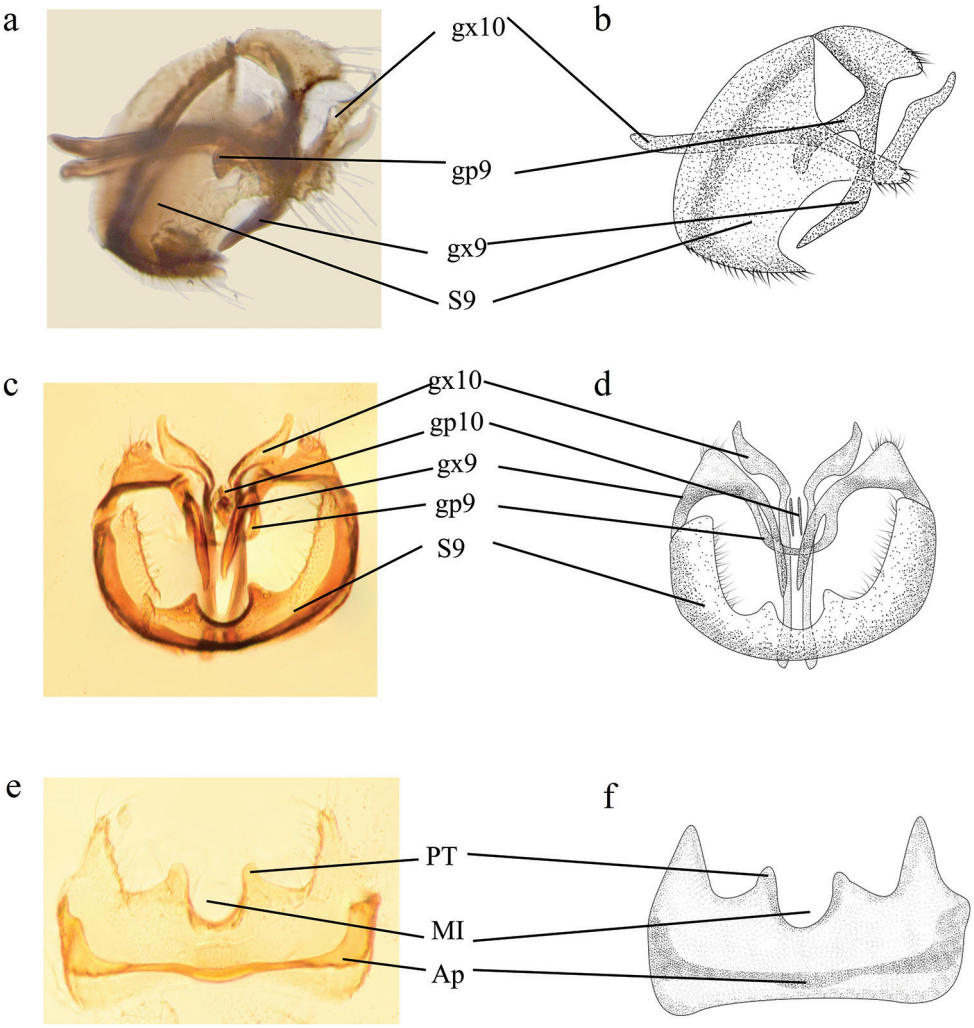


**Figure 6.** *Coniopteryx* (*Coniopteryx*) *alticola* Sziráki, 2002, male genitalia **a, b** genitalia, lateral view **c, d** genitalia, caudal view **e, f** Sternite 9 (S9), ventral view.

(Province): Yuanjiang (County): Jiangdong Park, [23.6001°N, 102.0098°E], 18.iii.2019, leg. Yaru Zhao (CAU).

**Measurements.** Forewing length 1.7 mm, width 0.9 mm. Hindwing length 1.4 mm, width 0.6 mm.

**Redescription. Male:** ***Head*** (Fig. 5a–c). Frons with prominent anterior process. Antennae brown, 25-segmented, 1.0 mm in length. Basal flagellomeres two times as long as broad. Subsequent flagellomeres tapering gradually. Apical flagellomere almost as long as wide.

***Thorax.*** Light brown. Meso- and metanotum with dorsal dark spots. Legs yellowish brown.

***Wing.*** Wing membrane light greyish brown, almost hyaline.

***Male terminalia*** (Fig. 6a–f). Accord with the description by Sziráki (2002).

**Remarks.** *Coniopteryx* (*Coniopteryx*) *alticola* Sziráki, 2002 belongs to the *C. lobifrons* species group (Sziráki 2004). The members of this group are characterized by the presence of a prominent process on the frons and of a protuberance on the first flagellomere (Fig. 5b, c). *Coniopteryx* (*C.*) *alticola* was originally described from Thailand (Sziráki 2002) and the examined specimens represent the first record of this species from China.

**Distribution.** China, Yunnan, first record; Thailand.

***Coniopteryx* (*Coniopteryx*) *tenuisetosa* sp. nov.**

http://zoobank.org/95D212F4-D6D2-4F6C-8A1E-FC7C7073F128

Figs 7, 8

**Type material.** ***Holotype*** 1 male, China: Tibet (Province): Linzhi (City), [29.6019°N, 94.4168°E], 8.vi.2019, leg. Yaru Zhao (CAU). ***Paratypes*** 39 males and 54 females, same data as holotype (CAU).

**Other material.** 2 males, China: Yunnan (Province): Lincang (City): Fengqing (County), [24.5934°N, 99.9001°E], 23.iv.1981, leg. Chikun Yang (CAU). 1 male, China: Yunnan (Province): Baoshan (City): Tengchong (County), [25.0199°N, 98.4800°E], 25.iv.1981, leg. Chikun Yang (CAU). 1 male, China: Yunnan (Province): Ruili (County): Mengxiu (Township), [25.0667°N, 98.4167°E], 2.v.1981, leg. Chikun Yang (CAU). 3 males, China: Yunnan (Province): Ruili (County): Mengxiu (Township): Nanjingli (Village), [24.0917°N, 97.8460°E], 2.v.1981, leg. Fasheng Li (CAU). 5 males, China: Tibet (Province): Linzhi (City): Linzhi (County): Gengzhang (Township), [29.7298°N, 94.0870°E], 1.vi.1978, leg. Fasheng Li (CAU). 1 male, China: Tibet (Province): Linzhi (City): Linzhi (County), [29.6019°N, 94.4168°E], 3.vi.1978, leg. Fasheng Li (CAU). 1 male, China: Tibet (Province): Linzhi (City): Bomi (County): Yigong (Township), [30.2389°N, 94.8523°E], 28.vi.1978, leg. Fasheng Li (CAU). 2 males, China: Tibet (Province): Linzhi (City): Bomi (County): Zhamu (Township), [29.7103°N, 95.5857°E], 1.vii.1978, leg. Fasheng Li (CAU). 1 male, China: Tibet (Province): Linzhi (City): Milin (County), [29.0428°N, 93.8898°E], 4.vi.1978, leg. Fasheng Li (CAU). 1 male, China: Tibet (Province): Linzhi (City): Lulang (County), [29.8208°N, 94.7382°E], 2.viii.1978, leg. Fasheng Li (CAU). 2 males, China: Tibet (Province): Linzhi (City): Chayu (County), [29.7103°N, 95.5857°E], 2.viii.1978, leg. Fasheng Li (CAU). 7 males, China: Tibet (Province): Linzhi (City): Milin (County), [29.0423°N, 94.2364°E], 9.vi.2019, leg. Yaru Zhao (CAU).

**Diagnosis.** Male genitalia: median apical incision shallow, U-shaped, less than half of sternite 9 length; terminal process blunt in lateral view; distal part of gonocoxites 10 short and stout, with tiny hairs.

**Measurements.** Forewing length 2.0–2.8 mm, width 1.0–1.3 mm. Hindwing length 1.5–1.7 mm, width 0.5–0.7 mm.

**Description. Male:** ***Head*** (Fig. 7a, b). Brown. Frons without projections. Compound eyes large. Antennae brown, 28-segmented, 1.2–1.5 mm in length. Scape and pedicel

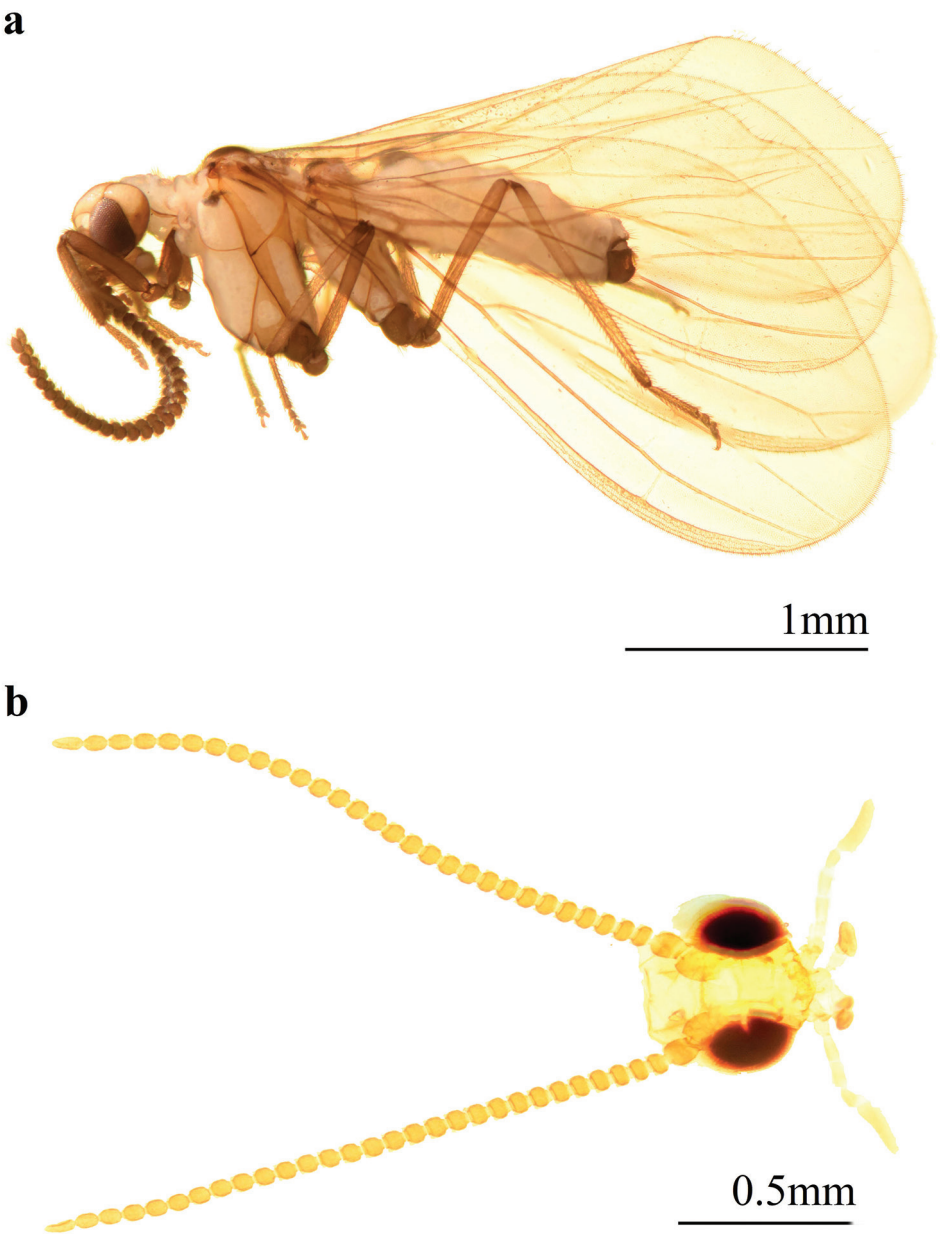


**Figure 7.** *Coniopteryx* (*Coniopteryx*) *tenuisetosa* sp. nov., male **a** habitus, lateral view **b** head, dorsal view.

broad and blunt. Basal flagellomeres wider than long, distal flagellomeres gradually tapering toward apex, apical flagellomere almost as long as wide. Apices of flagellomeres covered with scattered scale-like hairs and two whorls of setae. Maxillary and labial palps brown.

***Thorax.*** Yellowish brown. Meso- and metanotum dorsal dark spots. Legs yellowish brown, except the brown coxae.

***Wing.*** Wing membrane light greyish brown, almost hyaline.

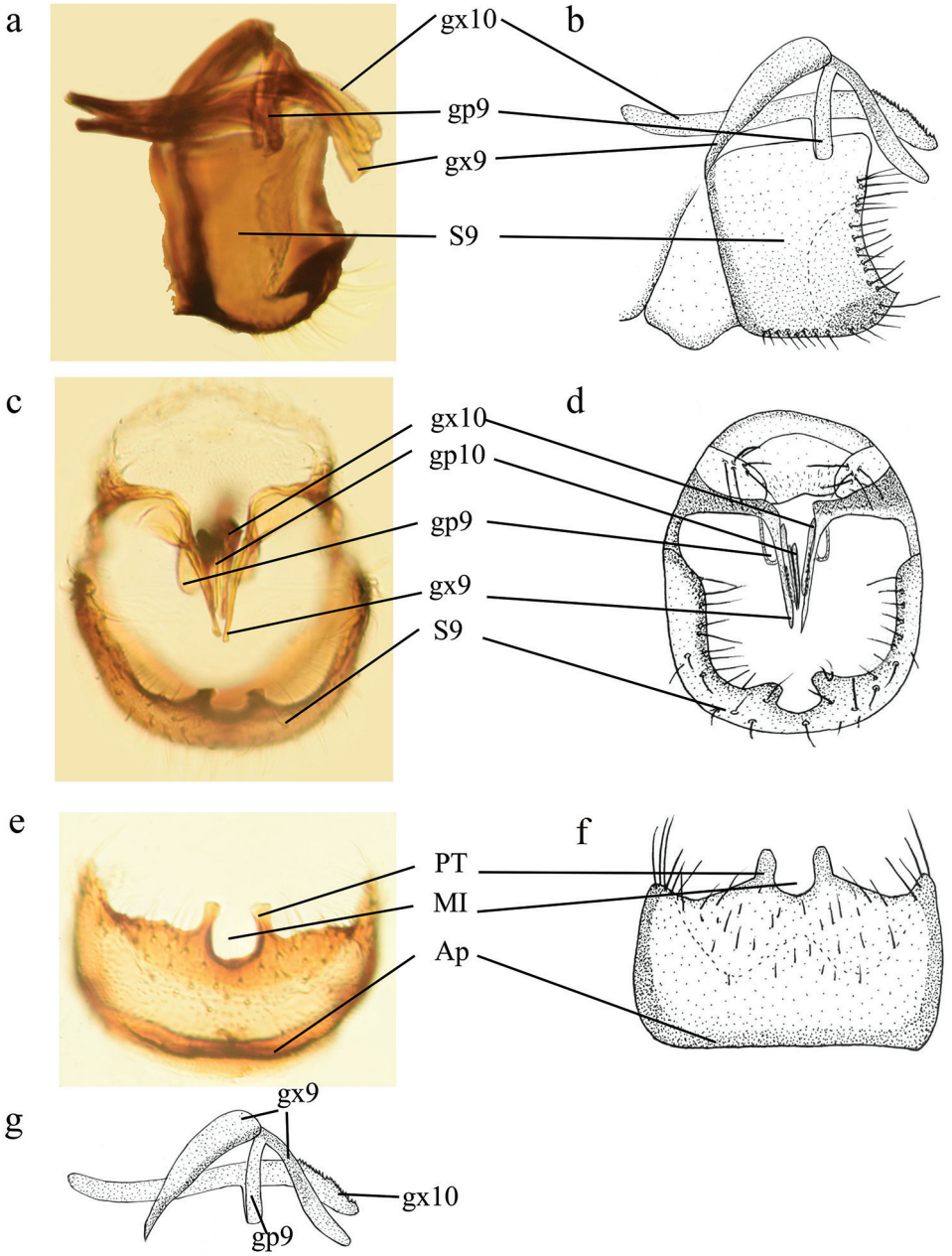


**Figure 8.** *Coniopteryx* (*Coniopteryx*) *tenuisetosa* sp. nov., male genitalia **a, b** genitalia, lateral view **c, d** genitalia, caudal view **e, f** sternite 9, ventral view **g** gonocoxites 10 (gx10), gonocoxites 9 (gx9) and gonapophyses 9 (gp9), lateral view.

***Male terminalia*** (Fig. 8a–g). Sternite 9 higher than wide in lateral view; anterior margin straight laterally; ventral apodeme along anterior margin not interrupted; lateral process rounded and blunt; terminal process short and acute in lateral view,

rounded and blunt in caudal view; median apical incision shallow and U-shaped, and its depth less than half the length of the sternite 9. Gonocoxites 10 long and slender, bent downwards near apex, distal portion serrated and covered with many tiny setae. Gonapophyses 10 as a pair of long, slender rods.

**Distribution.** China (Tibet, Yunnan).

**Etymology.** The species name *tenuisetosa* "thin-haired" is a composed adjective of Latin derivation, referring to the thin setae on the distal portion of gonocoxites 10.

**Remarks.** The new species is similar to *Coniopteryx* (*Coniopteryx*) *aspoecki* Kis, 1967, but the two species differ in configuration of the male genitalia. In particular, *Coniopteryx* (*Coniopteryx*) *tenuisetosa* is characterized by a short, not prominent terminal process of sternite 9 in lateral view, while it is prominent and arched in *C. aspoecki*. Moreover, in the new species, the distal portion of gonocoxites 10 is relatively robust and serrated, while in *C. aspoecki* it is thin, apically tapered and smooth.

***Coniopteryx* (*Coniopteryx*) *serrata* sp. nov.**

http://zoobank.org/5779FE7C-048C-49D6-9218-88C254002379

Figs 9, 10

**Type material.** ***Holotype*** 1 male, China: Yunnan (Province): Puer (City): Meizihu Park, [22.7551°N, 100.9845°E], 20.iii.2019, leg. Yaru Zhao. ***Paratype*** 1 male, same data as holotype (CAU).

**Other material.** 1 male, China: Yunnan (Province): Ruili (County): Mengxiu (Township), [25.0667°N, 98.4167°E], 2.v.1981, leg. Chikun Yang (CAU). 1 male, China: Yunnan (Province): Puer (City): Simao (District), [22.7860°N, 100.9798°E], 7.vi.1981, leg. Chikun Yang (CAU). 3 males, China: Yunnan (Province): Ruili (County): Mengxiu (Township): Tuanjiezhai (Village), [24.0917°N, 97.8460°E], 30.iii.2019, leg. Yaru Zhao (CAU).

**Diagnosis.** Male genitalia: median apical incision V-shaped. Its depth is more than the half of the length of sternum 9. Terminal process long and acute in lateral view. Distal part of gonocoxites 10 bent upwards perpendicularly.

**Measurements.** Forewing length 2.2–2.4 mm, width 0.8–1.1 mm. Hindwing length 1.5–1.8 mm, width 0.7–0.8 mm.

**Description. Male:** ***Head*** (Fig. 9a, b). Yellowish brown. Frons without projections. Compound eyes large. Antennae brown, 27–28-segmented, 1.2 mm in length. Scape and pedicel long and narrow. Basal flagellomeres two times wider than long, apical flagellomeres tapered. Flagellomeres scattered with scale-like setae at apex and two circles of hair-like sensilla; setae present on most segments except basal ones. Maxillary and labial palps yellowish brown.

***Thorax.*** Brown. Meso- and metanotum with dorsal dark spots. Legs yellowish brown except the brown coxae.

***Wing.*** Wing membrane light greyish brown, almost hyaline.

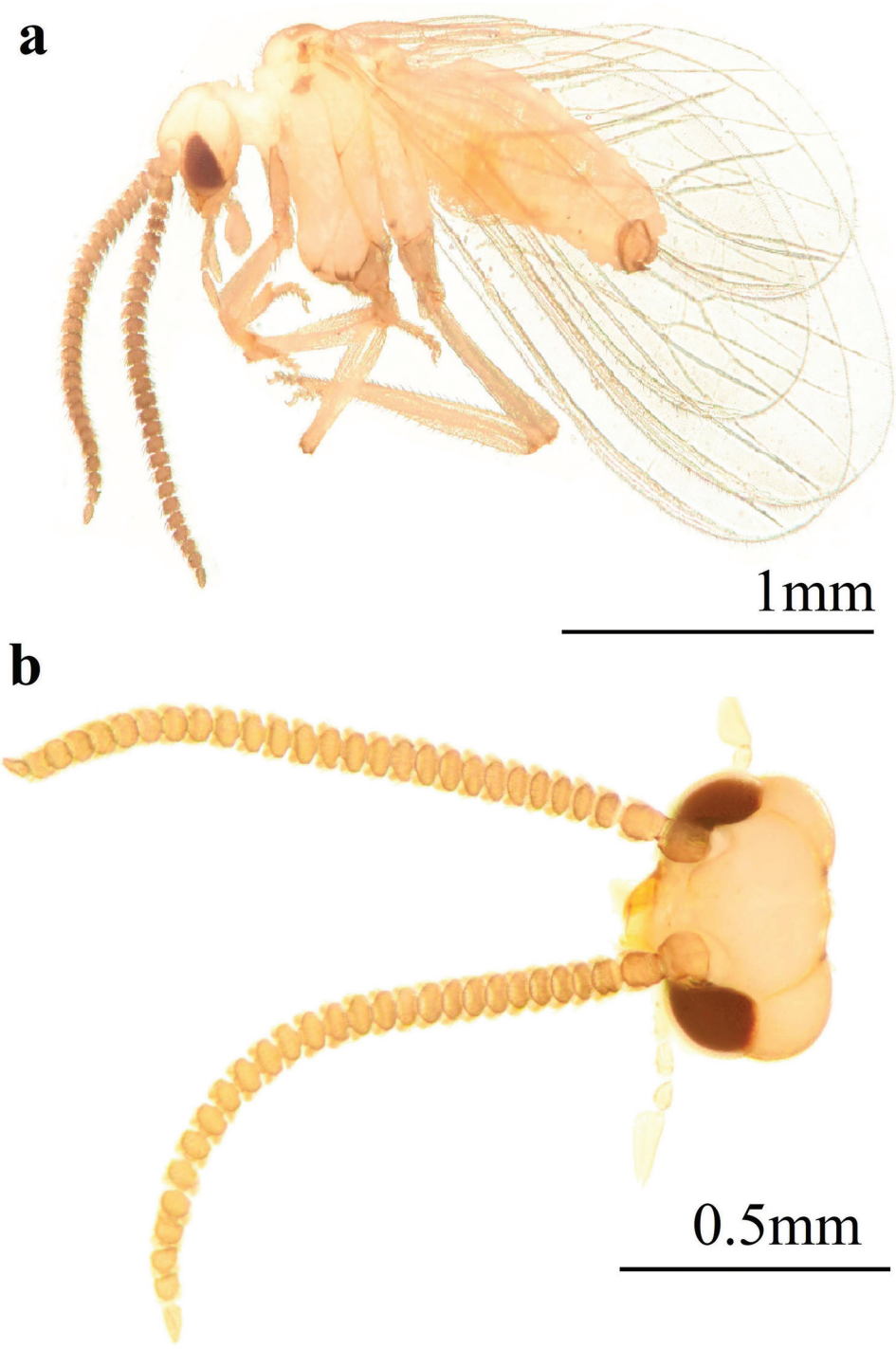


**Figure 9.** *Coniopteryx* (*Coniopteryx*) *serrata* sp. nov., male **a** habitus, lateral view **b** head, dorsal view.

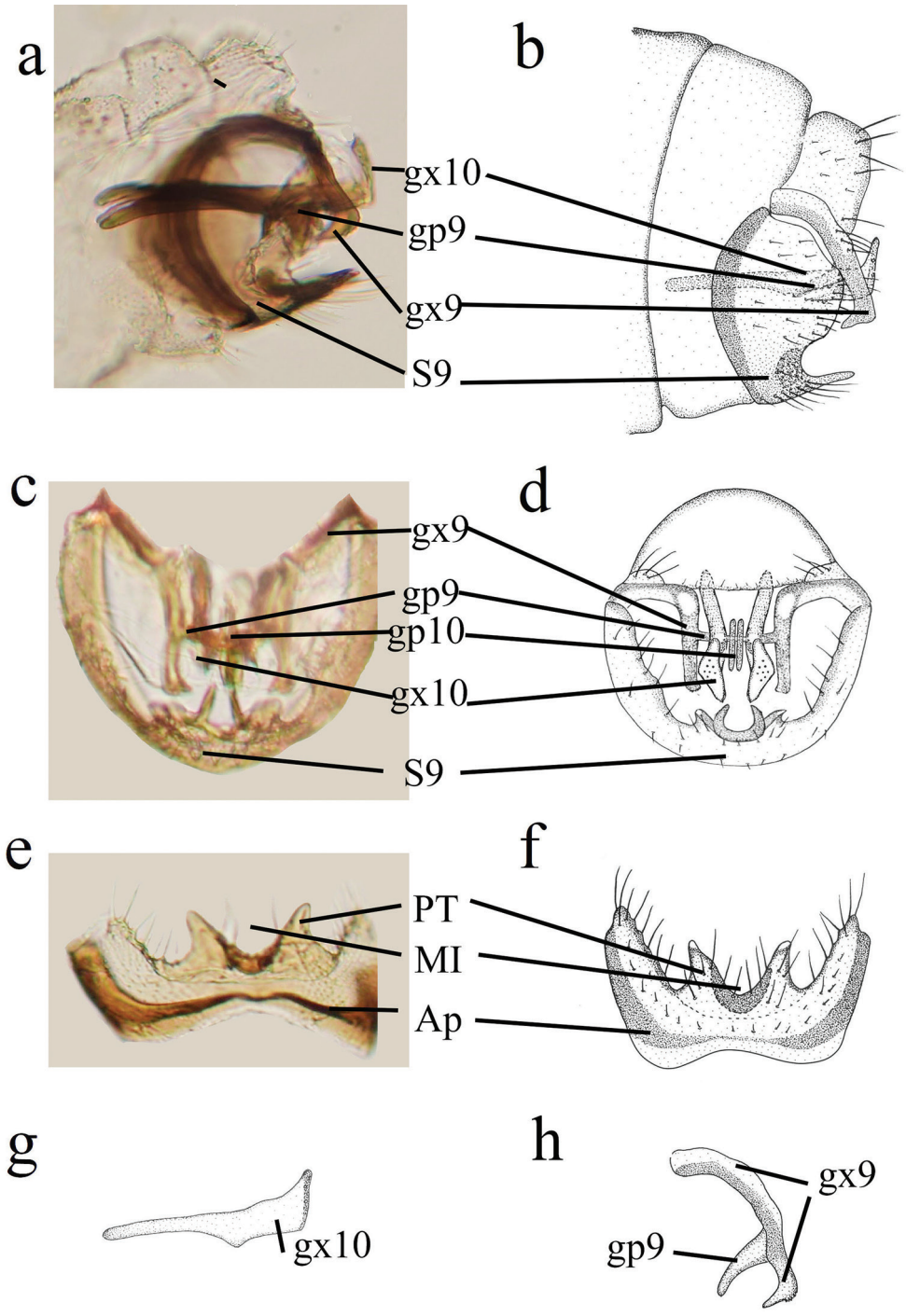


**Figure 10.** *Coniopteryx* (*Coniopteryx*) *serrata* sp. nov., male genitalia **a, b** genitalia, lateral view **c, d** genitalia, caudal view **e, f** sternite 9, ventral view **g** gonocoxites 10, lateral view.

***Male terminalia*** (Fig. 10a–h). Sternite 9 slightly higher than wide in lateral view; anterior margin arched in lateral view; apodeme along anterior margin wide, but interrupted or very thin ventrally; lateral process rounded and blunt; terminal process slender and acute in lateral view; median apical incision V-shaped with two short appendages in the middle. Gonocoxites 9 long and sinuated, distal section directed forwards perpendicularly and serrated. Gonocoxites 10 long and slender, bent upward distally, ventral process small. Gonapophyses 10 as a pair of long, slender rods.

**Distribution.** China (Yunnan).

**Etymology.** The species name is a Latin adjective referring to the minute serrations on the distal portion of gonocoxite 9.

**Remarks.** The genitalia of the new species suggest a close relationship with *Coniopteryx* (*Coniopteryx*) *wuyishana* Yang & Liu, 1999. However, the two species differ in the shape of the sternite 9. The new species is characterized by having a V-shaped median apical incision while it is U-shaped in *C.* (*C.*) *wuyishana*. Moreover, in *Coniopteryx* (*Coniopteryx*) *serrata* the anterior margin of sternite 9 stretches forwards laterally and the apodeme along the anterior margin is very thin and interrupted ventrally. In contrast, *C.* (*C.*) *wuyishana* is characterized by a straight anterior margin of sternite 9, and a ventrally complete anterior apodeme of sternite 9.

## Acknowledgements

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# The taxonomic status of *Myotis nesopolus larensis* (Chiroptera, Vespertilionidae) and new insights on the diversity of Caribbean *Myotis*

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## Abstract

*Myotis nesopolus* currently comprises two subspecies. The nominate subspecies (*M. n. nesopolus*) occurs on the Caribbean islands of Curaçao and Bonaire, Netherlands Antilles, whereas *M. n. larensis* is known from mainland South America in northeastern Colombia and northwestern Venezuela. Our Maximum Likelihood phylogenetic analyses of cytochrome-b gene sequences recovered *M. nesopolus* as a paraphyletic group, with *M. n. nesopolus* and *M. n. larensis* as non-sister lineages. The haplotype network indicates that these two subspecies do not share any haplotypes and are in different evolutionary trajectories. Additionally, these two subspecies can be distinguished on the basis of qualitative and quantitative morphological traits. This pattern supports the recognition of *M. nesopolus* and *M. larensis* as full species. Our results also reveal that the assemblage of Caribbean *Myotis* do not form a monophyletic group. Caribbean species are phylogenetically close to mainland species from northern South America and Central America, suggesting that colonization of Caribbean islands happened multiple times.

**Resumo**

Atualmente *Myotis nesopolus* compreende duas subespécies: *M. n. nesopolus* ocorre nas ilhas caribenhas de Curaçao e Bonaire, Antilhas Holandesas, enquanto *M. n. larensis* é conhecido para o continente da América do Sul, no nordeste da Colômbia e noroeste da Venezuela. Nossa inferência filogenética por Máxima Verossimilhança recuperou *M. nesopolus* como parafilética, com *M. n. nesopolus* e *M. n. larensis* sendo linhagens não-irmãs. Além disso, essas duas subespécies não compartilham nenhum haplótipo. Adicionalmente, as subespécies podem ser diferenciadas a partir de caracteres morfológicos e morfométricos. Esse achado suporta o reconhecimento de *M. nesopolus* e *M. larensis* como espécies distintas. Nossos resultados revelam que os *Myotis* do Caribe não formam um grupo monofilético. Espécies caribenhas são filogeneticamente próximas de espécies continentais das Américas Central e do Sul, sugerindo que a colonização das ilhas do Caribe aconteceu por múltiplos eventos de dispersão.

**Keywords**

Bats, biogeography, Lesser Antilles, morphology, morphometry, taxonomy, South America, Venezuela

## Introduction

*Myotis* Kaup, 1829 (Vespertilionidae, Myotinae) comprises more than 120 species distributed worldwide, and is the most speciose genus of bats (Simmons 2005; Burgin et al. 2018). Twenty-seven species are recognized from the Neotropics (Wilson 2008; Moratelli et al. 2017, 2019a; Carrión-Bonilla and Cook 2020). However, molecular evidence has revealed that the current species richness is underestimated (Claire et al. 2011; Larsen et al. 2012a; Chaverri et al. 2016; Moratelli et al. 2017).

Two subspecies of *Myotis nesopolus* Miller, 1900 are recognized. The nominate subspecies, *M. n. nesopolus*, is known from Curaçao and Bonaire in the Netherlands Antilles. The other subspecies, *M. n. larensis* LaVal, 1973, is known from mainland South America in northeastern Colombia and northwestern Venezuela (LaVal 1973; Wilson 2008; Muñoz-Garay and Mantilla-Meluk 2012; Moratelli et al. 2013). LaVal (1973) described *Myotis larensis* as a full species from "Río Tocuyo, Lara, Venezuela". Genoways and Williams (1979), however, treat *larensis* as a subspecies of *Myotis nesopolus*. Miller's (1900) description of *M. nesopolus* was based on one specimen from Willemstad, Curaçao, Netherlands Antilles. Subsequently, Genoways and Williams (1979) considered that representatives of *Myotis* from Bonaire island, originally identified as *Myotis nigricans* (Schinz, 1821), were misidentifications of *M. nesopolus*, which was confirmed by Moratelli et al. (2017).

Previous molecular and morphological studies questioned the subspecific status of mainland populations of *M. nesopolus*, suggesting that the two subspecies might represent different species (Larsen et al. 2012b; Moratelli et al. 2013, 2017). Here we reassess the taxonomic status of *M. n. larensis* in the light of new morphological and genetic analyses.

## Materials and methods

### Specimens examined

Specimens of *M. nesopolus* used in this study are deposited in the American Museum of Natural History (**AMNH**, New York, USA), Carnegie Museum of Natural History (**CM**, Pittsburgh, USA), Smithsonian's National Museum of Natural History (**USNM**, Washington DC, USA), and Museum of Texas Tech University (**TTU**, Lubbock, USA). We examined the holotype of *M. n. nesopolus* (USNM 101849), two topotypes from Curaçao (CM 52432, USNM 105128), and nine specimens from Bonaire (Appendix 1). Material of *M. n. larensis* includes the holotype (AMNH 130709), and fifteen additional specimens from mainland Venezuela.

### Molecular analyses

Phylogenetic analyses of complete cytochrome-b gene (cyt-b, 1,140 bp, no gaps) sequences were conducted for the Neotropical assemblage of *Myotis*. A total of 122 sequences, including outgroups, were retrieved from GenBank (Appendix 2). We used the palearctic species *Myotis brandtii* (Eversmann, 1845) and *Myotis gracilis* Ognev, 1927 as outgroups because they are sister to the Neotropical clade (see Ruedi et al. 2013). Multiple sequence alignment of full length cyt-b sequences were performed with MEGA X (Kumar et al. 2018), using MUSCLE algorithm with default settings (Edgar 2004). Subsequently, the Bayesian Information Criterion (BIC), as implemented in JModelTest2 (Darriba et al. 2012), was used to determine the best-fit models of nucleotide substitution. The Hasegawa-Kishino-Yano model (Hasegawa et al. 1985) was chosen to correct the heterogeneity rate using gamma-distribution with invariant sites (i.e., HKY + Γ + I).

The phylogenetic analysis was carried out using Maximum Likelihood (ML) method (Felsenstein 1981), in the software RAxML v8.0 (Stamatakis 2014). To assess the nodal support, we calculated a nonparametric bootstrap using 1000 replications. Genetic distance values for cyt-b sequences were calculated in MEGA X using the Kimura 2-parameter model (Kimura 1980).

To understand the population structure of *M. n. nesopolus*, *M. n. larensis* and other phylogenetically related population groups, we built a haplotype network (distribution of haplotypes by previously defined population groups) using the median-joining algorithm in the Network 4.6.1.3 software (Bandelt et al. 1999).

### Morphological and morphometric analyses

We examined 284 specimens for the morphological comparisons, including *M. n. nesopolus* ($N = 10$), *M. n. larensis* ($N = 9$) and 14 species of Neotropical *Myotis* deposited in 11 collections in Brazil, Canada and United States (Appendix 1). Specimens were

identified following Wilson (2008) and Moratelli et al. (2011, 2013, 2017). The main qualitative morphological characters used in the comparisons were: (i) presence and height of sagittal crest; (ii) presence and height of lambdoidal crests; (iii) inclination shape of the frontal and parietal bones; (iv) presence of a fringe of hairs along the trailing edge of the uropatagium; (v) dorsal and ventral fur texture and height; (vi) pattern of fur coloring, with the capitalized color nomenclature following Ridgway (1912).

We took one external and 16 craniodental measurements (Table 1), using digital calipers to the nearest 0.01 mm. Measurements were made under binocular microscopes with low magnification (usually 6×). Measurements were recorded from adults and are reported in millimeters (mm). The length of ear and body mass were recorded from skin labels. We used a principal component analysis (PCA) to identify general trends of cranial size and shape variation among samples, and a discriminant function analysis (DFA), with a priori identification of samples, to compare skull size and shape of *M. n. nesopolus* (*N* = 9) and *M. n. larensis* (*N* = 9). For these analyses, we selected a subset of 11 craniodental dimensions representing different axes of the length and width of skull, rostrum, and mandible, as follows: greatest length of skull, including incisors (GLS), condylo-incisive length (CIL), mastoid breadth (MAB), braincase breadth (BCB), interorbital breadth (IOB), postorbital breadth (POB), breadth across canines (BAC), breadth across molars (BAM), maxillary toothrow length (MTL), molariform toothrow length (M1–M3), and mandibular toothrow length (MAN). PCA and DFA analyses were run in R software (R Development Core Team 2012) using the MASS and Lattice packages (Venables and Ripley 2002; Sarkar 2008). Because multivariate procedures require complete data sets, missing values (ca 1.5% of the total dataset) were estimated from the existing raw data using the Amelia II package (Honaker

**Table 1.** Description of cranial, mandibular, and external dimensions (and their abbreviations). Lengths were measured from the anteriormost point or surface of the 1st structure to the posteriormost point or surface of the 2nd structure, except as specified.

| Measurements | Acronyms | Descriptions |
|---|---|---|
| Forearm length | FA | From the elbow to the distal end of the forearm including carpals |
| Greatest length of skull | GLS | From the apex of the upper internal incisors, to the occiput |
| Condylo-canine length | CCL | From the anterior surface of the upper canines to a line connecting the occipital condyles |
| Condylo-basal length | CBL | From the premaxillae to a line connecting the occipital condyles |
| Condylo-incisive length | CIL | From the apex of upper internal incisors to a line connecting the occipital condyles |
| Basal length | BAL | Least distance from the apex of upper internal incisors to the ventral margin of the foramen magnum |
| Zygomatic breadth | ZYG | Greatest breadth across the outer margins of the zygomatic arches |
| Mastoid breadth | MAB | Greatest breadth across the mastoid region |
| Braincase breadth | BCB | Greatest breadth of the globular part of the braincase |
| Interorbital breadth | IOB | Least breadth between the orbits |
| Postorbital breadth | POB | Least breadth across frontals posterior to the postorbital bulges |
| Breadth across canines | BAC | Greatest breadth across outer edges of the crowns of upper canines, including cingulae |
| Breadth across molars | BAM | Greatest breadth across outer edges of the crowns of upper molars |
| Maxillary toothrow length | MTL | From the upper canine to M3 |
| Molariform toothrow length | M1–M3 | From M1 to M3 |
| Mandibular length | MAL | From the mandibular symphysis to the condyloid process |
| Mandibular toothrow length | MAN | From the lower canine to m3 |

et al. 2011) implemented in R software. Measurements were transformed to natural logs and covariance matrices were computed considering all variables. Subsequently, an analysis of variance using Mann-Whitney statistics was employed to test whether the population samples differ in cranial dimensions. The comparison was made using *p*-values and when less than 0.001 were considered as statistically significant. This analysis was run in the software PAST 3.3 (Hammer et al. 2001).

## Results

### Molecular analyses

The ML phylogeny based on cyt-b sequences indicates that *M. nesopolus*, as currently recognized, is paraphyletic, with *M. n. nesopolus* more closely related to an eastern Peruvian unidentified lineage, whereas *M. n. larensis* was recovered more closely related to an unidentified lineage from western Ecuador (Fig. 1), although this phylogeny and branching events has low nodal support. These unidentified species from Peru and Ecuador were originally designated as *Myotis nigricans* by the original collector due to morphological similarities. However, *M. nigricans* has been recovered as polyphyletic and considered a cryptic species complex in many studies (Moratelli et al. 2011, 2013, 2016, 2017; Larsen et al. 2012a). Therefore, we decided not to give a name to the lineages related to *M. nesopolus* and *M. larensis*. We emphasize that the previous identification of these specimens as *M. nigricans* by one of our authors (RJL) in a previous study (Larsen et al. 2012a) indicates that these populations are morphologically distinct from those considered here as *M. nesopolus* and *M. larensis*.

The Caribbean *Myotis* species do not form a monophyletic group, being related to *Myotis atacamensis* (Lataste, 1892) and other mainland putative species. Nevertheless, the phylogenetic relationship of Caribbean *Myotis* clade is not fully resolved, since a polytomy was recovered among *M.* sp. 3 from Honduras and the ancestral lineage of *M. n. nesopolus* and *M.* sp. 2 from Peru, and of *M. n. larensis* and *M.* sp. 1 from Ecuador. Similarly, a polytomy was recovered among *M. atacamensis*, *M. martiniquensis* and an ancestral lineage of *M. dominicensis*, *M. nyctor* and *M.* sp. 4 from Suriname (Fig. 1).

The average cyt-b pairwise distance between *M. n. larensis* and *Myotis* sp. 1 from western Ecuador is 2.1% ± 0.3; between *M. n. nesopolus* and *Myotis* sp. 2 from eastern Peru is 3.8% ± 0.4; and between *M. n. nesopolus* and *M. n. larensis* is 4.0% ± 0.3 (Table 2). Levels of intraspecific variation were less than 0.8% for all recognized and putative species (Table 2).

The haplotype network indicates that there are no haplotypes shared between *M. n. nesopolus*, *M. n. larensis*, and phylogenetically close species (Fig. 2). The haplotypes were grouped into small clusters well-distributed among the populations, with no central haplotype. The network indicates spatial structuring with isolation among the population groups tested, agreeing with what was obtained by phylogenetic inference.

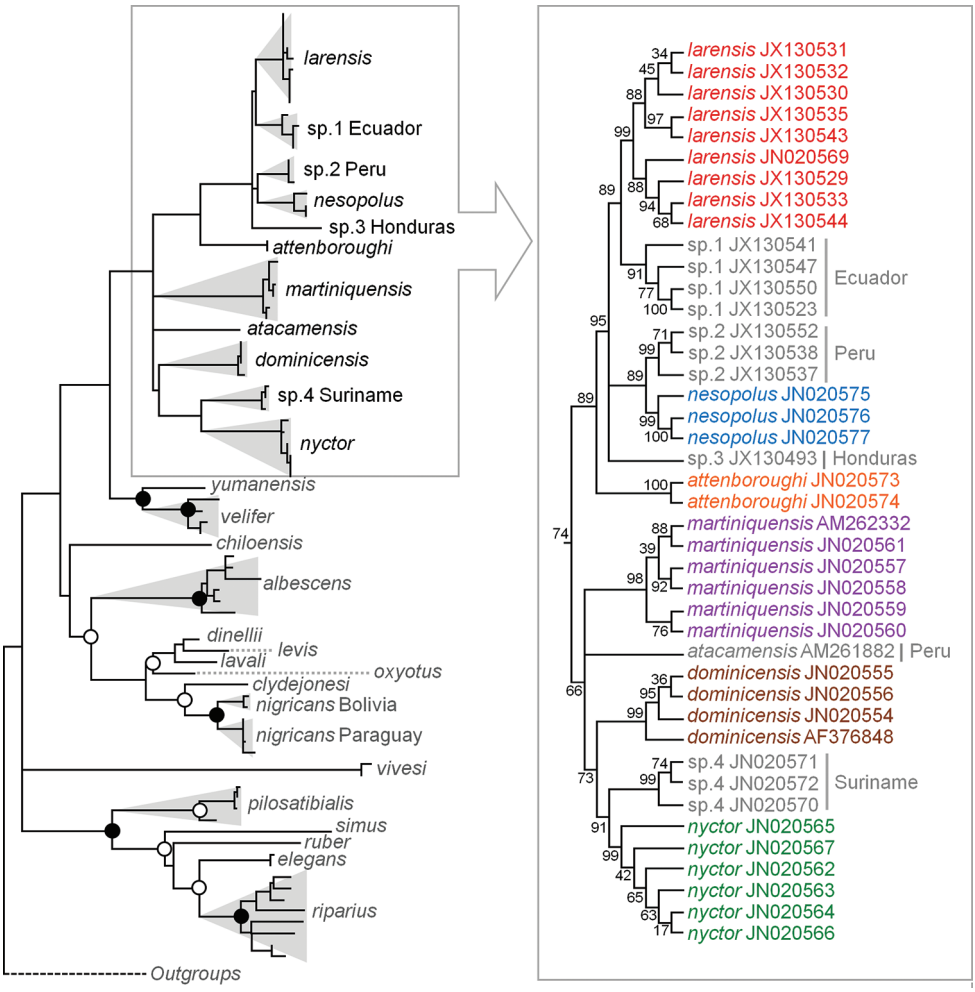


**Figure 1.** Phylogenetic tree resulting from the Maximum Likelihood analysis of cytochrome-b sequences of species of *Myotis*. Nodal support was calculated by bootstrap and black solid circles are values between 100–95% and hollow white circle are values between 94–90%. Values less than 90% were not indicated. The rectangle encloses the phylogenetic relationship, where branches were transformed to cladogram, among *M. nesopolus*, *M. larensis*, Caribbean *Myotis* (colored terminals) and mainland haplogroups of five more closely related species and candidate species.

## Morphological analyses

The first principal component (PC1) accounted for 87% of the total craniometric variation, and represents overall skull size (Fig. 3A, B). Along this axis, scores of *M. n. larensis* and *M. n. nesopolus* do not overlap. On the other hand, the two samples overlap broadly along the second principal component (PC2 = 5%) which represents overall skull shape. The distribution of *M. n. larensis* and *M. n. nesopolus* samples across size and shape axes in the discriminant analysis (Fig. 3C, D) is similar to that observed in

**Table 2.** Average Kimura 2-parameter genetic distances within (along diagonal) and among (below diagonal) *Myotis* taxa based on cytochrome-b gene sequences. Boldface value indicates the distance between *M. larensis* and *M. nesopolus*. Hyphen indicates groups with a single sequence.

| | Taxa | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *M. atacamensis* (Peru) | – | | | | | | | | | | | |
| 2 | *Myotis* sp. 4 (Suriname) | 0.085 | 0.002 | | | | | | | | | | |
| 3 | *M. nyctor* (Grenada) | 0.103 | 0.080 | – | | | | | | | | | |
| 4 | *M. nyctor* (Barbados) | 0.089 | 0.070 | 0.002 | 0.004 | | | | | | | | |
| 5 | *M. dominicensis* (Dominica) | 0.080 | 0.087 | 0.092 | 0.088 | 0.001 | | | | | | | |
| 6 | *M. martiniquensis* (Martinique) | 0.087 | 0.093 | 0.089 | 0.094 | 0.887 | 0.002 | | | | | | |
| 7 | *M. n. larensis* (Venezuela) | 0.093 | 0.107 | 0.127 | 0.119 | 0.097 | 0.096 | 0.003 | | | | | |
| 8 | *Myotis* sp. 1 (W Ecuador) | 0.091 | 0.104 | 0.134 | 0.120 | 0.092 | 0.093 | 0.021 | 0.002 | | | | |
| 9 | *Myotis* sp. 2 (E Peru) | 0.104 | 0.115 | 0.138 | 0.126 | 0.107 | 0.104 | 0.034 | 0.033 | 0.001 | | | |
| 10 | *M. n. nesopolus* (Bonaire) | 0.103 | 0.115 | 0.147 | 0.124 | 0.104 | 0.106 | **0.040** | 0.044 | 0.038 | 0.008 | | |
| 11 | *Myotis* sp. 3 (Honduras) | 0.103 | 0.116 | 0.133 | 0.120 | 0.107 | 0.105 | 0.046 | 0.049 | 0.056 | 0.053 | – | |
| 12 | *M. attenboroughi* (Tobago) | 0.081 | 0.093 | 0.101 | 0.099 | 0.091 | 0.088 | 0.068 | 0.075 | 0.076 | 0.078 | 0.079 | 0.000 |

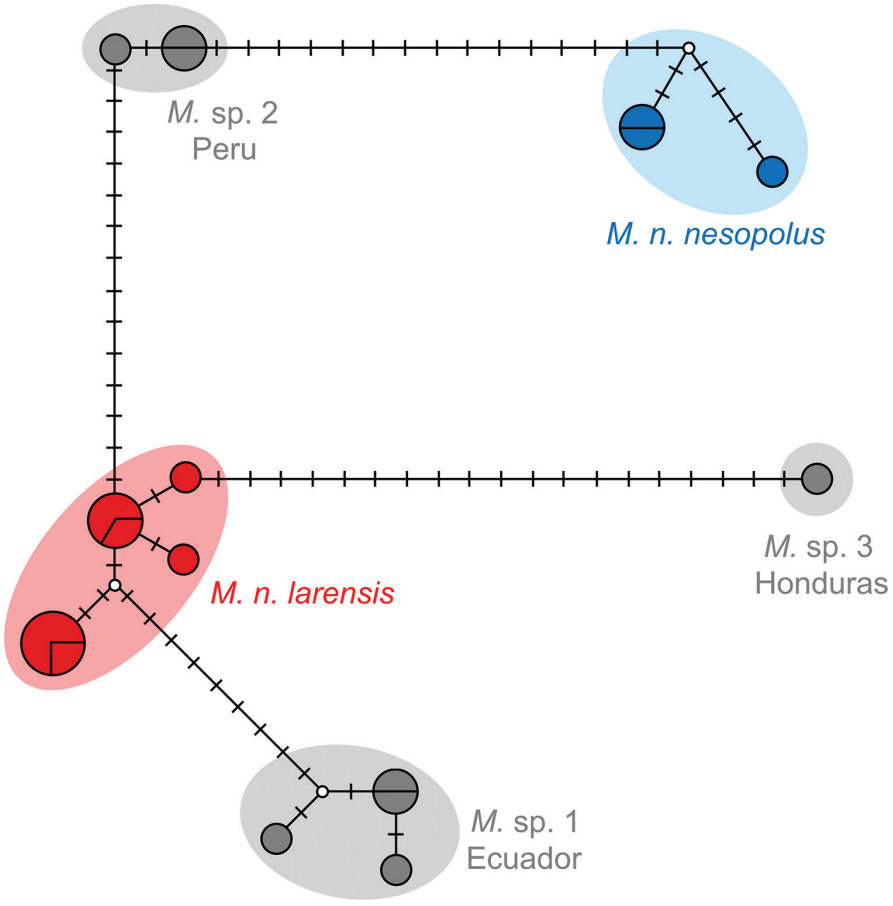


**Figure 2.** Haplotype network from cyt-b sequences of *Myotis nesopolus* (blue), *Myotis larensis* (red) and other mainland closest *Myotis* lineages from Central and South America. Each tick mark represents a single base-pair mutation.

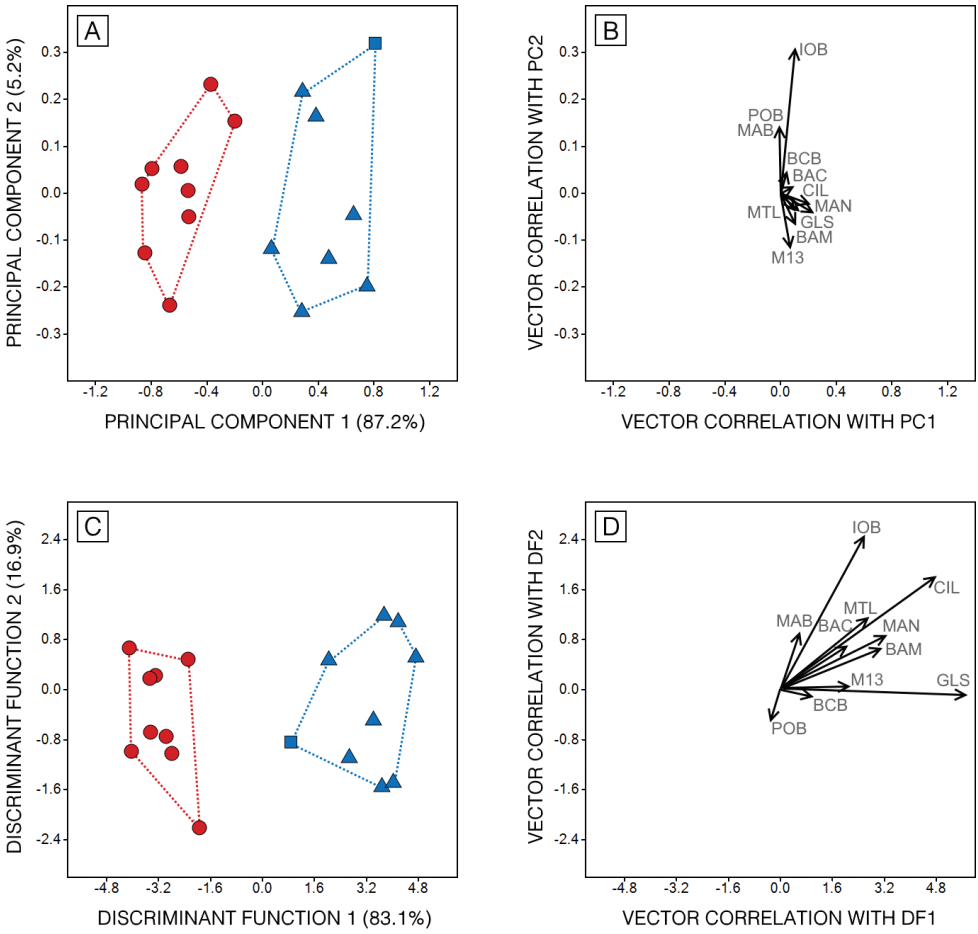


**Figure 3.** Plots showing convex-hulls and vector correlation of cranial measurements of Principal Component Analysis (**A, B**) and Discriminant Function Analysis (**C, D**) for *Myotis nesopolus* from Curaçao (black square), *Myotis nesopolus* from Bonaire (blue triangles) and *Myotis larensis* from Venezuela mainland (red dots).

the PCA. Measurements associated with skull and mandible length (GLS, CIL, MAN) and skull width (IOB) were the most useful to discriminate samples (Table 3). Considering that skull axes are represented by the set of measurements used in the morphometric multivariate analysis, these results reveal that *M. n. larensis* and *M. n. nesopolus* have distinct skull size and shape.

Populations from the Antilles and mainland South America do not overlap in measurements of several characters, which may be useful in distinguishing species: *M. n. larensis* forearm length ranges from 31.2 to 33.2 mm, and GLS from 13.6 to 14.5 mm; *M. n. nesopolus* forearm length ranges from 28.2 to 31.0 mm, and GLS from 12.9 to 13.4 mm. The Mann-Whitney test found significant differences in 11 of the 14 measurements tested (Table 4).

Population samples from the Antilles and mainland South America have several qualitative morphological differences. Specimens of *M. n. nesopolus* have moderately

**Table 3.** Vector correlation loadings with original variables of principal components (PC1 and PC2) and discriminant functions (DF1 and DF2) for selected samples of *M. larensis* and *M. nesopolus*. See Table 1 for variable abbreviations.

| Measurements | PC 1 | PC2 | DF1 | DF2 |
|---|---|---|---|---|
| MAN | 0.324 | -0.091 | 0.063 | 0.016 |
| GLS | 0.573 | -0.103 | 0.109 | 0.026 |
| CIL | 0.506 | -0.056 | 0.093 | 0.027 |
| MAB | 0.097 | 0.327 | 0.012 | 0.012 |
| BCB | 0.109 | 0.108 | 0.019 | 0.003 |
| IOB | 0.258 | 0.775 | 0.051 | 0.014 |
| POB | -0.02 | 0.363 | -0.005 | 0.026 |
| BAC | 0.198 | 0.031 | 0.04 | 0.021 |
| BAM | 0.277 | -0.165 | 0.059 | -0.015 |
| MTL | 0.262 | -0.088 | 0.052 | 0.011 |
| M1–3 | 0.187 | -0.298 | 0.040 | -0.007 |

**Table 4.** Selected measurements (mm) of *M. larensis* from Venezuela and *M. nesopolus* from Curaçao and Bonaire. Descriptive statistics include the mean, range (in parentheses), and sample size. See Table 1 for variable abbreviations. Mann-Whitney Test *p*-values was used to compare cranial measurements between samples. Measurements with hyphen (–) not were tested due to disparate samples size.

| Measurements | *Myotis larensis* | *Myotis nesopolus* | *P*–value |
|---|---|---|---|
| FA | 32.2 (31.2–33.2) 7 | 29.7 (28.2–31.0) 11 | – |
| GLS | 13.7 (13.3–14.4) 9 | 12.9 (12.8–13.1) 9 | < 0.001 |
| CCL | 12.1 (11.5–12.7) 9 | 11.6 (11.4–11.8) 9 | < 0.001 |
| CBL | 12.8 (12.4–13.5) 9 | 12.2 (12.0–12.5) 9 | < 0.001 |
| CIL | 12.9 (12.6–13.6) 9 | 12.4 (12.2–12.6) 9 | < 0.001 |
| BAL | 11.6 (11.2–12.4) 9 | 11.1 (10.9–11.3) 9 | < 0.001 |
| ZYG | 8.1 (8.0–8.2) 3 | 7.8 (7.7–8.0) 8 | – |
| MAB | 5.3 (5.1–5.6) 9 | 6.7 (6.4–6.8) 9 | 0.247 |
| BCB | 6.2 (6.1–6.3) 9 | 6.1 (5.9–6.2) 9 | 0.017 |
| IOB | 4.4 (4.0–4.7) 9 | 4.0 (3.9–4.2) 9 | 0.003 |
| POB | 3.3 (3.2–3.4) 9 | 3.3 (3.2–3.5) 9 | 0.374 |
| BAC | 3.3 (3.2–3.5) 9 | 3.0 (3.0–3.2) 9 | < 0.001 |
| BAM | 5.3 (5.1–5.5) 9 | 4.9 (4.8–5.0) 9 | < 0.001 |
| MTL | 5.2 (5.0–5.4) 9 | 4.8 (4.7–4.9) 9 | < 0.001 |
| M1M3 | 2.9 (2.8–3.2) 9 | 2.7 (2.6–2.8) 9 | < 0.001 |
| MAL | 9.8 (9.5–10.3) 4 | 9.0 (8.8–9.2) 9 | – |
| MAN | 5.5 (5.3–5.9) 8 | 5.1 (4.9–5.3) 9 | < 0.001 |

silky fur (length of dorsal fur 5–6 mm; length of ventral fur 3–4 mm); dorsal fur Dresden-Brown with little contrast between bases and tips slightly lighter tips; ventral fur with blackish bases and Light-Buff tips (Fig. 4A). Specimens of *M. n. larensis* have long silky fur (length of dorsal fur 6–8 mm; length of ventral fur 5–6 mm); dorsal fur strongly bicolored, with blackish bases (2/3) and Tawny-Olive tips (1/3); ventral fur with blackish bases and whitish tips (Fig. 4B). The sagittal crest is absent in *M. n. nesopolus*, the lambdoidal crests are generally absent or very low, and the parietal is inclined forward. Sagittal and lambdoidal crests are present in *M. n. larensis*, ranging from low to moderate in development, and the parietal is not inclined forward. In both populations, the second upper premolar (P3) is aligned in the toothrow and visible in labial view, and the occipital region is always rounded (Fig. 5).


**Figure 4.** Dorsal (left) and ventral (right) fur of a specimen of *Myotis nesopolus* (CM 52217 [**A**]) from Bonaire and the holotype of *Myotis larensis* (USNM 441737 [**B**]) from Lara, Venezuela.

The congruence between the molecular and morphological evidence indicates that the two subspecies of *M. nesopolus* do not form a clade. Thus, *M. larensis* represents an independent evolutionary lineage and should be treated as a full species.

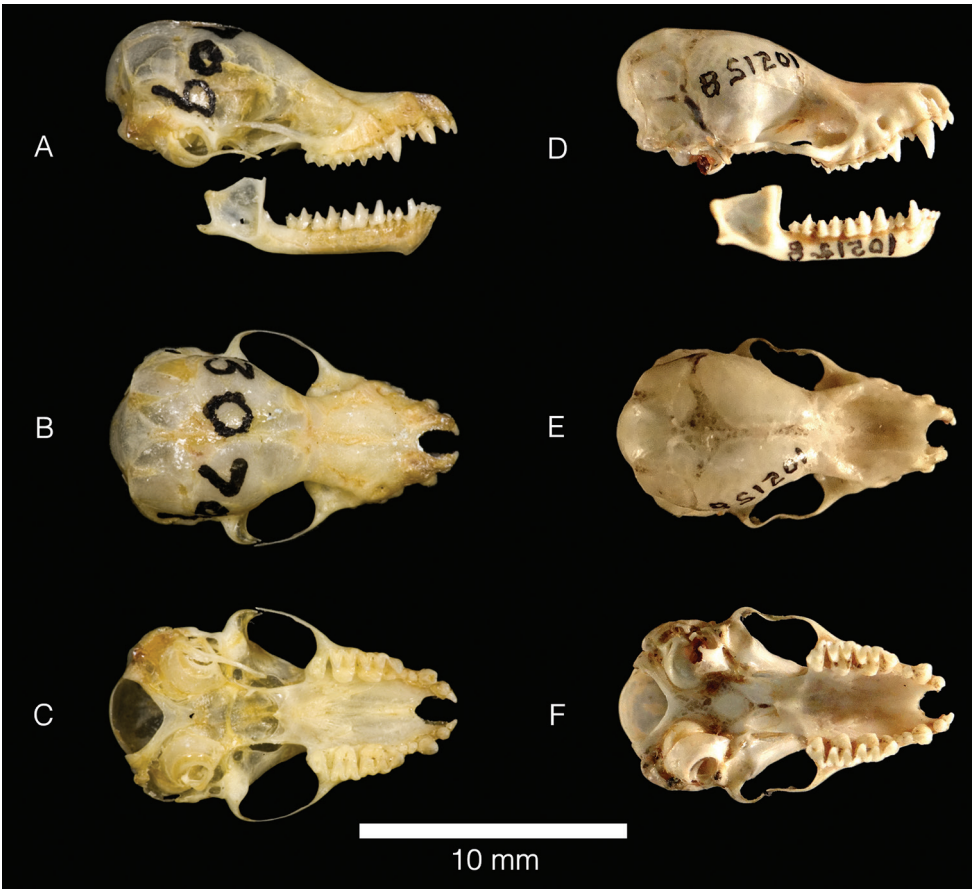


**Figure 5.** Skull profiles of *Myotis larensis* (AMNH 130709 [holotype]) from Venezuela in lateral (**A**), ventral (**B**) and dorsal (**C**) views; and *Myotis nesopolus* (USNM 105128 [topotype]) from Curaçao in lateral (**D**), ventral (**E**) and dorsal (**C**) views. The image of the *M. nesopolus* skull was inverted.

## Description and comparisons

*Myotis larensis* is a small-sized bat (total length 78–82 mm; forearm length 31.2–33.2; body mass 3–5 g), morphologically similar to several Neotropical congeners. Ears are moderate in size (length 10–13 mm), and when laid forward extend halfway from eye to nostril. Antitragal notch is barely evident. Membranes are Mummy-brown. Fur on dorsal surface of uropatagium extends slightly past the knees. Plagiopatagium is attached to the foot at toes level by a broad band of membrane. Third metacarpal, tibia, and skull are long in relation to forearm (mean ratios 0.96, 0.48, and 0.43, respectively; see LaVal (1973)).

*Myotis larensis* can be distinguished from all Caribbean and South American congeners by qualitative and quantitative traits. It differs from *M. nesopolus* by its larger size (no overlapping in forearm length and greatest length of skull), presence of sagittal crest, and dorsal fur longer and strongly bicolored. Considering the *Myotis* species

that occurs in the northern South America, *M. larensis* differs from *M. albescens* (É. Geoffroy, 1806) by the absence of a fringe of hairs along the trailing edge of the uropatagium; from *M. keaysi* J. A. Allen, 1914, *M. pilosatibialis* LaVal, 1973, *M. riparius* Handley, 1960, and *M. simus* Thomas, 1901 by the long silky dorsal fur strongly bicolored. *Myotis larensis* can also be distinguished from *M. simus* by the plagiopatagium broadly attached at base of the toes. *Myotis larensis* differs from *M. diminutus* Moratelli & Wilson, 2011 by its larger cranial dimensions and dorsal fur strongly contrasting; from *M. handleyi* Moratelli et al., 2013 by its strongly contrasting and long silk dorsal fur and shorter forearm; from *M. oxyotus* (Peters, 1867) by having a smaller skull, less steeply sloping frontals and strongly contrasting dorsal fur. *Myotis larensis* differs from *M. attenboroughi* Moratelli et al., 2017 by its lighter and strongly contrasting dorsal fur and larger skull; and from *M. clydejonesi* Moratelli et al., 2016 by its moderate steeply sloping frontals, less inflated braincase, smaller skull and dorsal fur strongly contrasting. *Myotis larensis* differs from *M. caucensis* Allen, 1914 by its smaller skull and strongly contrasting dorsal fur. *Myotis larensis* can be distinguished from *M.* cf. *nigricans* from northern South America (sensu Moratelli et al. 2013) by the lighter dorsal and ventral fur, more developed sagittal and lambdoid crests and parietal not inclined forward.

## Discussion

Genoways and Williams (1979) determined that mainland and island specimens of *M. larensis* and *M. nesopolus*, respectively, were morphometrically similar, with Venezuelan specimens slightly smaller than those from Curaçao. As a result, they recognized *M. larensis* as a subspecies of *M. nesopolus*, which was followed by subsequent authors (e.g., Simmons 2005; Wilson 2008; Moratelli et al. 2019b). However, our results do not support this arrangement, indicating a morphometric discontinuity and qualitative morphological differences between *M. larensis* and *M. nesopolus*.

Previous phylogenetic studies based on mitochondrial and nuclear DNA recovered *M. nesopolus* and *M. larensis* as sister lineages and questioned the subspecific status of *M. larensis* because the cyt-b genetic distance of 4% between mainland and Antilles populations suggests a potential for separation at the species level (see Bradley and Baker 2001; Larsen et al. 2012b). However, this study did not include the mainland samples from Ecuador and Peru. Our phylogenetic analyses revealed that *M. nesopolus* and *M. larensis* are not sister lineages and do not share haplotypes. The genetic distances between *M. nesopolus*, *M. larensis* and their sister species are greater than 2%. About this, Bradley and Baker (2001) indicate that genetic distance values between 2 and 11% from cyt-b sequences had a high probability of being indicative of conspecific populations or valid species and merit additional study concerning specific status. Our investigation found a conspicuous phenotypic discontinuity in variation of both the size and shape of the skull and other external characters. Thus, the strong congruence between the morphological, morphometric and molecular evidence presented here supports the hypothesis that *M. larensis* represents a full species.

Nevertheless, it is important to mention the limitation of cyt-b gene for establishing species boundaries in the Caribbean clade, particularly between *M. larensis* and *M.* sp. 1 from Ecuador and between *M. nesopolus* and *M.* sp. 2 from Peru. Although widely used (e.g., Larsen et al. 2012a, b; Moratelli et al. 2016, 2017; Carrión-Bonilla and Cook 2020), the application of cyt-b data to species delimitation and inference of phylogenetic relationships in *Myotis* from the Caribbean clade was insufficient. This demonstrates the need to expand the use of new genetic markers for future systematic studies with the Caribbean *Myotis* assemblage.

With the recognition of *M. larensis* at the species level hierarchy, *M. nesopolus* is restricted to Bonaire and Curaçao and is the only species of the genus found in these islands (Fig. 6). Similarly, other Caribbean islands have unique *Myotis* species, including: *Myotis dominicensis* Miller, 1902 restricted to Dominica and Guadeloupe; *Myotis martiniquensis* LaVal, 1973 is restricted to Martinique; *Myotis attenboroughi* is restricted to Tobago; and *Myotis nyctor* LaVal & Schwartz, 1974 is restricted to Barbados and Grenada (LaVal 1973; Larsen et al. 2012a; Moratelli et al. 2017). However, the taxonomic status of some populations of these species needs to be reassessed. For example, *Myotis nyctor* was described from Barbados and subsequently recorded from Grenada (LaVal 1973; LaVal and Schwartz 1974; Moratelli et al. 2017). Although our phylogenetic analysis grouped the samples of *M. nyctor* from Barbados ($N$ = 5) and Grenada ($N$ = 1) in the

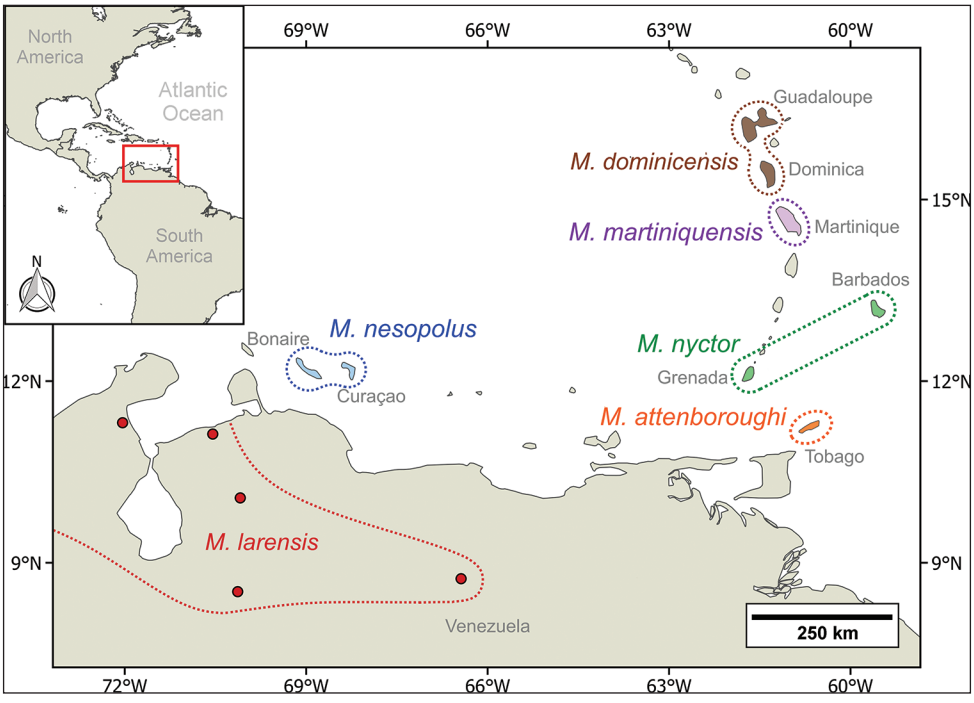


**Figure 6.** Geographic distributions of *Myotis larensis* (restricted to mainland South America in Venezuela and Colombia) and Caribbean *Myotis* species *M. nesopolus*, *M. dominicensis*, *M. martiniquensis*, *M. nyctor*, and *M. attenboroughi*.

same clade (Fig. 1), and with low genetic distance between them (ca 0.2%; Table 2), there are qualitative and quantitative morphological differences between specimens from these two islands (see Larsen et al. 2012a). The similarity in the cyt-b sequences between Grenada and Barbados specimens may be explained by the retained ancestral polymorphism due to the very recent separation (Stadelmann et al. 2007; Larsen et al. 2012a).

The biogeographic interpretations made by Larsen et al. (2012b) suggest at least two independent *Myotis* invasions into the Lesser Antilles, and reverse colonization by Caribbean *Myotis* to mainland Central and South America—the latter being a well-documented pattern in other Caribbean bat lineages (Dávalos 2005, 2006, 2010; Genoways et al. 2005; Pavan and Marroig 2017; Tavares et al. 2018). In addition, some biogeographic and ecological aspects suggest the need for taxonomic revision of some species. The distance and geographic isolation between Barbados and Grenada (ca 255 km) are greater than between Dominica and Martinique (ca 42 km), each one having a unique *Myotis* species. Moreover, Barbados and Grenada are separated by the Tobago Basin, with an ocean depth of approximately 2500 m and no ridges that may have connected these two populations during glaciation periods (Speed 1981; Humphrey 1997; Graham 2003). Considering the apparent low vagility and the small home range of *Myotis* in general (e.g., LaVal and Fitch 1977; Castella et al. 2001; Moratelli et al. 2019b), it is possible that the populations of *M. nyctor* from these two islands are isolated and on different evolutionary trajectories. The same rationale might be valid for *M. dominicensis*, where the populations from Guadeloupe and Martinique are isolated by approximately 42 km of sea. However, there are several oceanic ridges between these two islands, which may have served as bridges connecting these two populations during the last glaciation (Speed 1981; Humphrey 1997; Graham 2003). Thus, we suggest that future studies on systematics and biogeography of Caribbean *Myotis* should focus on the definition of the taxonomic status of island populations from Grenada and Guadeloupe.

With the recognition of *M. larensis* as a full species, 28 species of Neotropical *Myotis* (sensu Stadelmann et al. 2007) are currently recognized: *M. albescens* (É. Geoffroy, 1806), *M. ruber* (É. Geoffroy, 1806), *M. nigricans* (Schinz, 1821), *M. levis* (I. Geoffroy, 1824), *M. chiloensis* (Waterhouse, 1840), *M. oxyotus* (Peters, 1866), *M. atacamensis* (Lataste, 1892), *M. nesopolus* Miller, 1900, *M. simus* Thomas, 1901, *M. dinellii* Thomas, 1902, *M. dominicensis* Miller, 1902, *M. caucensis* Allen, 1914, *M. keaysi* J.A. Allen, 1914, *M. riparius* Handley, 1960, *M. elegans* Hall, 1962, *M. larensis* LaVal, 1973, *M. martiniquensis* LaVal, 1973, *M. pilosatibialis* LaVal, 1973, *M. nyctor* LaVal & Schwartz, 1974, *M. diminutus* Moratelli & Wilson, 2011, *M. lavali* Moratelli et al., 2011, *M. izecksohni* Moratelli et al., 2011, *M. handleyi* Moratelli et al., 2013, *M. midastactus* Moratelli & Wilson, 2014, *M. clydejonesi* Moratelli et al., 2016, *M. attenboroughi* Moratelli et al., 2017, *M. bakeri* Moratelli et al., 2019, and *M. armiensis* Carrión-Bonilla & Cook, 2020. However, our results indicate that there are at least four haplogroups that might correspond to undescribed species. This scenario confirms the Neotropical region as a highly diverse region for *Myotis*.

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## Appendix I

List of specimens examined in the American Museum of Natural History (AMNH, New York, USA); Carnegie Museum of Natural History (CM, Pittsburgh, USA); Field Museum of Natural History (FMNH, Chicago, USA), Louisiana State University, Museum of Zoology (LSUMZ, Baton Rouge, USA); Museu de Zoologia da Universidade de São Paulo (MZUSP, São Paulo, Brazil); Museum of Texas Tech University (TTU, Lubbock, USA); Museum of Vertebrate Zoology, University of California (MVZ, Berkeley, USA); National Museum of Natural History, Smithsonian Institution (USNM, Washington, D.C., USA); Natural History Museum of Los Angeles County (LACM, Los Angeles, USA); Natural History Museum, University of Kansas (KU, Lawrence, USA); and Royal Ontario Museum (ROM, Toronto, Canada). Specimens marked with asterisks were included in the morphometric multivariate analysis.

*Myotis albescens* (*N* = 10). Venezuela: Trujillo, Valera, Río Motatán (USNM 370933); Apure, Pto. Páez, Río Cinaruco (USNM 373913); Bolívar, Río Supamo, 50 km SE

El Manteco (USNM 387693); Miranda, 7 km E Río Chico, Nr. Pto. Tuy (USNM 387700); Amazonas, Capibara, 106 km SW Esmeralda, Brazo Casiquiare (USNM 409392, 409395); Amazonas, San Juan, 163 km ESE Pto. Ayacucho, Río Manapiare (USNM 409403, 409408, 409410, 409411).

*Myotis attenboroughi* (*N* = 13). Trinidad and Tobago: Tobago Island, Charlottesville, 1 km N of Pirate's Bay, Saint John Parish (USNM 540692 [paratype], 540693 [holotype]); Tobago Island, St. Mary Parish, Hillsborough Reservoir (USNM 538064, 538065, 538066, 538067, 538068, 538069, 540619, 540620, 540621, 540694, 540695 [paratypes]).

*Myotis caucensis* (*N* = 22): Colombia: Valle del Cauca, Cauca river (AMNH 32787 [holotype]); Valle del Cauca, Candelaria, Ingenio Mayangüez (USNM 461858–461867). Peru: Cuzco, Madre de Dios, 15 km E Puerto Maldonado, Reserva Cuzco Amazónico (KU 144288–144291); Loreto, Yarinacocha (LSUMZ 12252, 12254–12258).

*Myotis clydejonesi* (*N* = 1): Suriname: Sipaliwini, Raleigh Falls (TTU 109227 [holotype]).

*Myotis diminutus* (*N* = 2): Ecuador: Los Ríos, Santo Domingo, 47 Km S (By Road), Río Palenque Science Center (USNM 528569 [holotype]). Colombia: Nariño, La Guayacana (LACM 18761).

*Myotis handleyi* (*N* = 27). Venezuela: Araguá, Rancho Grande Biological Station, 13 km NW Maracay (USNM 517503, 562923, 562924, 562925, 562926–562933, 562934, 562935, 562936, 562937); Distrito Federal, Pico Ávila, 5 km NE Caracas, near Hotel Humboldt (USNM 370932 [holotype]); Distrito Federal, Pico Ávila, 5 km NE Caracas, near Hotel Humboldt (USNM 370891 [paratype]); Miranda, Curupao, 5 km NW Guarenas (USNM 387723); Monagas, 3 km NW Caripe, near San Agustín (USNM 409391, 409429–409431, 409433, 409435, 409437, 409438).

*Myotis keaysi* (*N* = 45). Venezuela: Araguá, Rancho Grande Biological Station, 13 km NW Maracay (USNM 370893–370895, 370898–370902, 370911–370913, 370915– 370922, 370924, 370926, 370929); Araguá, Rancho Grande Biological Station, 13 km NW Maracay (USNM 370927, 370928, 370930, 370931); Araguá, Pico Guayamayo, 13 km NW Maracay (USNM 521564); Araguá, Rancho Grande, Portachuelo (USNM 562920, 563005, 563006); Araguá, Rancho Grande (USNM 562921); Bolívar, Gran Sabana (USNM 130625, 130626); Carabobo, Montalban, 4 km NW Montalban, La Copa (USNM 441741, 441742); Distrito Federal, Los Venados, 4 km NW Caracas (USNM 370889); Distrito Federal, Pico Ávila, 5 km NNE Caracas, near Hotel Humboldt (USNM 370890); Distrito Federal, junction Puerto Cruz Highwayand Colonia Tovar Highway, 0.5 km W (USNM 562984); Guárico, Hacienda El Vira, 10 km NE Altagracia (USNM 387707); Miranda, San Andrés, 16 km SE Caracas (USNM 373920); Miranda, Curupao, 5 km NW Guarenas (USNM 387714–387716, 387718); Monagas, Caripe (USNM 534265).

*Myotis larensis* (*N* = 16). Venezuela: Lara, Río Tucuyo (AMNH 130709* [holotype]); Falcón, Capatárida, 6 km SSW (USNM 441710*, 441711*, 441728*, 441735*,

441736*, 441737*, 441740); Zulia, Nr. Cojoro, 35 km NNE Paraguaipoa (USNM 441721*). Guárico (TTU 48162, 48163, 48164, 48168, 48169, 48170); Barinas (CM 78645).

*Myotis nesopolus* (*N* = 26). Curaçao: Punda (USNM 101849 [holotype]); Willemstad, Scharloo (USNM 102158); Westpunt, 2.8 km S, 4.5 km E of (CM 52432, 5433*). Bonaire, 8.5 km N, 2 km Wkralendijk (CM 52203, 52204, 52205, 52206, 52207, 52208, 52209, 52211, 52212*, 52213, 52214, 52215, 52216*, 52217*, 52218*, 52219*, 52220*, 52221, 52222*, 52223*, 52224, 52225).

*Myotis* cf. *nigricans* (*N* = 23). Suriname: Para, Zanderij (CM 63933, 69053, 77699). Venezuela: Carabobo, Urama, 10 Km NW Urama, El Central (USNM 140447, 373921–373924, 373926, 373929, 373932, 373933, 373935, 373936, 373942, 373943, 373946, 373947, 373948, 373949, 373950, 441741, 441742).

*Myotis oxyotus* (*N* = 9). Venezuela: Amazonas, Cerro Duida, Cano Culebra, 50 km NW Esmeralda (USNM 405799); Amazonas, Cerro Neblina, Camp VII (USNM 560809–560811); Bolívar, Km. 125, 85 km SE El Dorado (USNM387712); Bolívar, El Pauji, 21 km NE Icabaru, El Pauji (USNM441750); Distrito Federal, Alto Ño León, 33 km SW Caracas (USNM 409427); Mérida, La Mucuy, 4 km E Tabay (USNM373919, 387705).

*Myotis pilosatibialis* (*N* = 11). Trinidad and Tobago: Trinidad Island, St. George (TTU 5441). Honduras: Francisco Morazán, 1 km W Talanga (LACM 36879 [holotype]). Guatemala: Chimaltenago, Chocoyos (FMNH 41653, 41839, 41840, 41841, 41843, 41844, 41845, 41846, 73365).

*Myotis riparius* (*N* = 33). Costa Rica: Puntarenas, 5.3 km S (byroad) San Vito (CM 92491); Limon, Fila La Maquina (LSUMZ 12928). French Guiana: Paracou, near Sinnamary (AMNH 266376, 268591). Guyana: Barima-Waini, North West District (USNM 568021); Potaro-Siparuni, Iwokrama Field Station, Iwokrama Forest (ROM 112049); Potaro-Siparuni, Iwokrama Reserve, Burro Burro River, 25 km WNW of Kurupukari (ROM 107278, 114620); Potaro-Siparuni, Mount Ayanganna, First Plateau Camp (ROM 114688, 114689); Upper Takutu-Upper Essequibo, Gunn'sStrip (ROM 106773). Nicaragua: Chontales (KU 11228). Panamá: Darién, Tacarcuna Village Camp, Río Pucro (USNM 310255 [holotype], 310254, 310256, 310257 [paratypes]); Darién, Rio Paya, Mouth (USNM 306798); Panamá, Cerro Azul (USNM 306795); Chiriquí (USNM 331916); Bocas del Toro, Isla Popa, 1 Km SE Deer Island Channel (USNM 464368). Trinidad and Tobago: Trinidad Island,St. George (TTU 5467). Venezuela: Amazonas,Boca Mavaca, 84 km SSE Esmeralda, 7 km up Río Mavaca (USNM 405803, 405804); Amazonas, Capibara, 106 km SW Esmeralda, Brazo Casiquiare (USNM 409457); Amazonas, ca 2 km SE Cerro Neblina Base Camp (USNM 560625); Amazonas, Tamatama, Río Orinoco (USNM 405806); Apure, Nulita, 29 km SW Santo Domingo, Selvas de San Camilo (USNM 416584, 441746, 441748); Araguá, Rancho Grande (USNM 562940); Barinas, 7 km NE Altamira (USNM 441743); Bolívar, Río Supamo, 50 km SE El Manteco (USNM 387721); Bolívar, San Ignacio de Yhuruani (USNM 448544).

*Myotis simus* (*N* = 56). Brazil: Amazonas, Borba (AMNH 91886–91892, 94224, 94225, 94227, 94230–94234); Amazonas, Itacoatiara (MZUSP 4372); Amazonas, Manaus (AMNH 79534, 91472–91478, 91500); Amazonas, Parintins (AMNH 92983, 93489–93497, 93922–93925); Amazonas, Rio Juruá (MZUSP 638, 1074).

## Appendix 2

Specimens used in cytochrome-b analyses, including terminal taxa (focal and putative species of *Myotis*), GenBank accession numbers of sequences, voucher specimens, localities of origin, and source of information. The information presented for terminal taxonomic identifications results from re-identification of specimens (see Materials and methods), and does not necessarily match those identifications assigned by researchers that generated the corresponding sequence(s) available at GenBank. Abbreviations and acronyms for institutional collections are as follows: American Museum of Natural History, New York, USA (AMNH), Carnegie Museum of Natural History, Pittsburg, USA (CM), Field Museum of Natural History, Chicago, USA (FMNH), Museum of Natural History, University of Kansas, Lawrence, USA (KU), Natural History Museum of Los Angeles County, Los Angeles, USA (LACM), Louisiana State University, Museum of Zoology, Baton Rouge, USA (LSUMZ), Museum of Vertebrate Zoology, University of California, Berkeley, USA (MVZ), University of Nebraska State Museum, Lincoln, USA (UNSM), Muséum national d'Histoire naturelle, Paris, France (MNHN), Národní Muzeum, Prague, Czech (NMP), Museo de Zoología de la Pontificia Universidad Católica del Ecuador, Quito, Ecuador (QCAZ), Royal Ontario Museum, Toronto, Canada (ROM), Universidad Autónoma Metropolitana, Iztapalapa, Mexico (UAMI), Universidade Federal Rural do Rio de Janeiro, Seropédica, Brazil (ALP); and Smithsonian National Museum of Natural History, Washington, DC, USA (USNM). Localities are arranged alphabetically by species and major political unities.

| Terminal | GenBank | Voucher | Locality | Source |
|---|---|---|---|---|
| *M. albescens* | JX130444 | CM 63920 | Nickerie, Suriname | Larsen et al. (2012a) |
| | JX130463 | TTU 85088 | Pastaza, Ecuador | Larsen et al. (2012a) |
| | JX130464 | TTU 85089 | Pastaza, Ecuador | Larsen et al. (2012a) |
| | JX130465 | TTU 85094 | Pastaza, Ecuador | Larsen et al. (2012a) |
| | JX130522 | TTU 85091 | Pastaza, Ecuador | Larsen et al. (2012a) |
| | JX130472 | TTU 102363 | El Oro, Ecuador | Larsen et al. (2012a) |
| | JX130500 | TTU 102348 | El Oro, Ecuador | Larsen et al. (2012a) |
| | JX130501 | TTU 103744 | Guayas, Ecuador | Larsen et al. (2012a) |
| | JX130445 | TTU 46343 | Huánuco, Peru | Larsen et al. (2012a) |
| | AF376839 | FMNH 162543 | Tarija, Bolivia | Ruedi and Mayer (2001) |
| | JX130503 | TTU 99124 | Boquerón, Paraguay | Larsen et al. (2012a) |
| | JX130502 | TTU 99801 | Ñeembucú, Paraguay | Larsen et al. (2012a) |
| | JX130504 | TTU 99818 | Ñeembucú, Paraguay | Larsen et al. (2012a) |
| *M. atacamensis* | AM261882 | MVZ 168933 | Olmos, Peru | Stadelmann et al. (2007) |
| *M. attenboroughi* | JN020573 | UNSM ZM–29470 | St. George Parish, Tobago | Larsen et al. (2012b) |
| | JN020574 | UNSM ZM–29483 | St. George Parish, Tobago | Larsen et al. (2012b) |
| *M. chiloensis* | AM261888 | – | Santiago, Chile | Stadelmann et al. (2007) |
| *M. clydejonesi* | JX130520 | TTU 109227 | Sipaliwini, Suriname | Larsen et al. (2012a) |
| *M. dinellii* | JX130475 | TTU 66489 | Córdoba, Argentina | Larsen et al. (2012a) |
| *M. dominicensis* | AF376848 | – | St. Joseph's Parish, Dominica | Ruedi and Mayer (2001) |
| | JN020554 | TTU 31519 | St. Joseph's Parish, Dominica | Larsen et al. (2012b) |
| | JN020555 | TTU 31507 | St. Joseph's Parish, Dominica | Larsen et al. (2012b) |
| | JN020556 | TTU 31508 | St. Joseph's Parish, Dominica | Larsen et al. (2012b) |

| Terminal | GenBank | Voucher | Locality | Source |
|---|---|---|---|---|
| *M. larensis* | JN020569 | TTU 48161 | Guárico, Venezuela | Larsen et al. (2012b) |
| | JX130529 | TTU 48162 | Guárico, Venezuela | Larsen et al. (2012a) |
| | JX130530 | – | Guárico, Venezuela | Larsen et al. (2012a) |
| | JX130531 | TTU 48163 | Guárico, Venezuela | Larsen et al. (2012a) |
| | JX130532 | TTU 48164 | Guárico, Venezuela | Larsen et al. (2012a) |
| | JX130533 | TTU 48168 | Guárico, Venezuela | Larsen et al. (2012a) |
| | JX130535 | CM 78645 | Guárico, Venezuela | Larsen et al. (2012a) |
| | JX130543 | TTU 48169 | Guárico, Venezuela | Larsen et al. (2012a) |
| | JX130543 | TTU 48169 | Guárico, Venezuela | Larsen et al. (2012a) |
| *M. lavali* | AF376864 | MVZ AD50 | Paraíba, Brazil | Ruedi and Mayer (2001) |
| *M. levis* | AF376853 | FMNH 141600 | São Paulo, Brazil | Ruedi and Mayer (2001) |
| *M. martiniquensis* | AM262332 | – | Martinique | Stadelmann et al. (2007) |
| | JN020558 | MNHN:2005–896 | Le Morne–Rouge, Martinique | Larsen et al. (2012b) |
| *M. martiniquensis* | JN020557 | MNHN:2005–895 | GrandRivière, Martinique | Larsen et al. (2012b) |
| | JN020559 | – | GrandRivière, Martinique | Larsen et al. (2012b) |
| | JN020560 | MNHN:2008–974 | GrandRivière, Martinique | Larsen et al. (2012b) |
| | JN020561 | – | GrandRivière, Martinique | Larsen et al. (2012b) |
| *M. nesopolus* | JN020575 | – | Bonaire, Netherlands Antilles | Larsen et al. (2012b) |
| | JN020576 | – | Bonaire, Netherlands Antilles | Larsen et al. (2012b) |
| | JN020577 | – | Bonaire, Netherlands Antilles | Larsen et al. (2012b) |
| *M. nigricans* | JX130450 | TTU 34952 | La Paz, Bolivia | Larsen et al. (2012a) |
| | JX130528 | TTU 34953 | La Paz, Bolivia | Larsen et al. (2012a) |
| | JX130455 | TTU 95992 | San Pedro, Paraguay | Larsen et al. (2012a) |
| | JX130496 | TTU 99743 | Presidente Hayes, Paraguay | Larsen et al. (2012a) |
| | JX130498 | TTU 99046 | Alto Paraguai, Paraguay | Larsen et al. (2012a) |
| | JX130499 | TTU 99802 | Ñeembucú, Paraguay | Larsen et al. (2012a) |
| | JX130539 | TTU 99516 | Concepción, Paraguay | Larsen et al. (2012a) |
| | JX130540 | TTU 99151 | Boquerón, Paraguay | Larsen et al. (2012a) |
| *M. nyctor* | JN020562 | CM 83427 | St. David Parish, Grenada | Larsen et al. (2012b) |
| | JN020563 | TTU 109225 | St. Thomas Parish, Barbados | Larsen et al. (2012b) |
| | JN020564 | TTU 109226 | St. Thomas Parish, Barbados | Larsen et al. (2012b) |
| | JN020565 | TTU 109229 | St. Thomas Parish, Barbados | Larsen et al. (2012b) |
| | JN020566 | TTU 109224 | St. Thomas Parish, Barbados | Larsen et al. (2012b) |
| | JN020567 | TTU 109230 | St. Thomas Parish, Barbados | Larsen et al. (2012b) |
| *M. oxyotus* | AF376865 | FMNH 129208 | Lima, Peru | Ruedi and Mayer (2001) |
| *M. pilosatibialis* | JX130449 | TTU 47514 | Yucatán, Mexico | Larsen et al. (2012a) |
| | JX130525 | – | Yucatán, Mexico | Larsen et al. (2012a) |
| | AF376852 | – | Yucatán, Mexico | Ruedi and Mayer (2001) |
| | JX130489 | CM 55764 | Vera Cruz, Mexico | Larsen et al. (2012a) |
| *M. elegans* | JX130479 | TTU 84380 | Atlántida, Honduras | Larsen et al. (2012a) |
| | JX130480 | TTU 84138 | Atlántida, Honduras | Larsen et al. (2012a) |
| *M. riparius* | AM261891 | – | La Selva, Costa Rica | Stadelmann et al. (2007) |
| | JX130474 | CM 78659 | Bolívar, Venezuela | Larsen et al. (2012a) |
| | JX130473 | CM 68443 | Para, Suriname | Larsen et al. (2012a) |
| | JX130469 | TTU 85344 | Esmeraldas, Ecuador | Larsen et al. (2012a) |
| | JX130515 | TTU 85345 | Esmeraldas, Ecuador | Larsen et al. (2012a) |
| | JX130572 | TTU 102681 | Esmeraldas, Ecuador | Larsen et al. (2012a) |
| | JX130492 | TTU 102883 | Esmeraldas, Ecuador | Larsen et al. (2012a) |
| | JX130513 | TTU 84870 | Pastaza, Equador | Larsen et al. (2012a) |
| | JX130506 | TTU 85090 | El Oro, Equador | Larsen et al. (2012a) |
| | JX130516 | QCAZ 11380 | Chimborazo, Equador | Larsen et al. (2012a) |
| | JX130436 | – | Huánuco, Peru | Larsen et al. (2012a) |
| | JX130481 | TTU 46348 | Huánuco, Peru | Larsen et al. (2012a) |
| | AF376866 | MVZ AD119* | Pernambuco, Brazil | Ruedi and Mayer (2001) |
| | AF376867 | MVZ AD472* | São Paulo, Brazil | Ruedi and Mayer (2001) |
| | AM262336 | – | São Paulo, Brazil | Stadelmann et al. (2007) |
| | JX130485 | TTU 99645 | Paraguari, Paraguay | Larsen et al. (2012a) |
| | JX130486 | TTU 94912 | Canindeyu, Paraguay | Larsen et al. (2012a) |

| Terminal | GenBank | Voucher | Locality | Source |
|---|---|---|---|---|
| *M. riparius* | JX130488 | TTU 122454 | Canindeyu, Paraguay | Larsen et al. (2012a) |
| | JX130491 | TTU 99378 | Canindeyu, Paraguay | Larsen et al. (2012a) |
| *M. velifer* | EF222340 | TTU 48587 | Texas, USA | Baird et al. (2008) |
| | EU680299 | TTU 44818 | Texas, USA | Baird et al. (2008) |
| | JX130468 | TTU 109261 | Texas, USA | Larsen et al. (2012a) |
| | AF376870 | MVZ 146766 | Sonora, Mexico | Ruedi and Mayer (2001) |
| | JX130478 | TTU 44816 | Tamaulipas, Mexico | Larsen et al. (2012a) |
| | JX130438 | UAMI 15306 | Michoacán, Mexico | Larsen et al. (2012a) |
| | JX130462 | UAMI 15304 | Michoacán, Mexico | Larsen et al. (2012a) |
| | JX130589 | UAMI 15305 | Michoacán, Mexico | Larsen et al. (2012a) |
| | JX130592 | – | Michoacán, Mexico | Larsen et al. (2012a) |
| | JX130477 | TTU 60983 | Santa Ana, El Salvador | Larsen et al. (2012a) |
| *M. vivesi* | AJ504406 | – | Gulf of California, Mexico | Stadelmann et al. (2004) |
| | AJ504407 | – | Gulf of California, Mexico | Stadelmann et al. (2004) |
| *M. yumanensis* | AF376875 | MVZ 15585 | California, USA | Ruedi and Mayer (2001) |
| *M.* sp. 1 | JX130523 | TTU 103803 | El Oro, Ecuador | Larsen et al. (2012a) |
| | JX130541 | TTU 103751 | El Oro, Ecuador | Larsen et al. (2012a) |
| | JX130546 | TTU 102760 | El Oro, Ecuador | Larsen et al. (2012a) |
| | JX130547 | TTU 102765 | El Oro, Ecuador | Larsen et al. (2012a) |
| | JX130548 | TTU 102487 | El Oro, Ecuador | Larsen et al. (2012a) |
| | JX130549 | TTU 102489 | El Oro, Ecuador | Larsen et al. (2012a) |
| | JX130550 | TTU 102490 | El Oro, Ecuador | Larsen et al. (2012a) |
| *M.* sp. 2 | JX130452 | TTU 46347 | Huánuco, Peru | Larsen et al. (2012a) |
| | JX130537 | TTU 46344 | Huánuco, Peru | Larsen et al. (2012a) |
| | JX130538 | TTU 46346 | Huánuco, Peru | Larsen et al. (2012a) |
| *M.* sp. 3 | JX130493 | TTU 61228 | Valle, Honduras | Larsen et al. (2012a) |
| *M.* sp. 4 | JN020570 | CM 63933 | Nickerie, Suriname | Larsen et al. (2012b) |
| | JN020571 | CM 69053 | Para, Suriname | Larsen et al. (2012b) |
| | JN020572 | CM 77699 | Para, Suriname | Larsen et al. (2012b) |
| **Outgroups** | | | | |
| *M. brandtii* | AF376844 | – | Neuhaus, Germany | Ruedi and Mayer (2001) |
| | AM261886 | NMP PB 916 | North west, Russia | Stadelmann et al. (2007) |
| | AY665139 | – | Moscow, Russia | Tsytsulina et al. (2012) |
| | AY665168 | – | Znojmo, Czech Republic | Tsytsulina et al. (2012) |
| *M. gracilis* | AB106609 | – | Hokkaido, Japan | Kawai et al. (2003) |
| | AB243025 | – | Hokkaido, Japan | Kawai et al. (2006) |
| | AB243026 | – | Hokkaido, Japan | Kawai et al. (2006) |
| | AB243027 | – | Hokkaido, Japan | Kawai et al. (2006) |
| | AB243028 | – | Hokkaido, Japan | Kawai et al. (2006) |
| | AB243029 | – | Hokkaido, Japan | Kawai et al. (2006) |
| | AB243030 | – | Hokkaido, Japan | Kawai et al. (2006) |