FOLIA PARASITOLOGICA 55: 277–290, 2008

Philometra sawara sp. n. and a redescription of *Philometra sciaenae* Yamaguti, 1941 and *Philometra nemipteri* Luo, 2001 (Nematoda: Philometridae): a morphological and molecular approach

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Key words: *Philometra sawara*, *Philometra sciaenae*, *Philometra nemipteri*, Nematoda, Philometridae, ITS2 rDNA, Japan, molecular study

Abstract. Morphological data and molecular analyses are used to describe the taxonomy of philometrid nematodes of the genus *Philometra* Costa, 1845, found in the gonads of marine fishes in Japan. A new *Philometra* species, *P. sawara* sp. n., is described based on male and female specimens collected from the gonads of *Scomberomorus niphonius* (Cuvier) (Japanese Spanish mackerel). Two additional species, *Philometra nemipteri* Luo, 2001 and *Philometra sciaenae* Yamaguti, 1941, are confirmed as valid species and are redescribed based on specimens collected from the gonads of *Nemipterus virgatus* (Houttuyn) (golden threadfin bream) and *Pennahia argentata* (Houttuyn) (silver croaker), respectively. Male *P. nemipteri* are first reported and described in this study. Redescriptions of female *P. nemipteri* and male and female *P. sciaenae* were also necessary based on our morphological observations. A molecular comparison of the internal transcribed spacer 2 (ITS2) region of the ribosomal DNA between *P. sawara*, *P. nemipteri*, *P. sciaenae*, and previously reported philometrid nematodes from the genus *Philometra* and *Philometroides* Yamaguti, 1935 supports the conclusion that the three *Philometra* species in the current study are independent. An ITS2-derived neighbour-joining tree, consisting of both the current specimens and previously described *Philometra* and *Philometroides* species, is also presented.

Majority of nematode species from the superfamily Dracunculoidea Stiles, 1907 are poorly described both biologically and taxonomically. These nematodes have been found in freshwater, brackish-water, and marine fishes. The use of light microscopy (LM) alone is insufficient for the identification of these species, especially for nematodes from the family Philometridae. Taxonomic examination requires the use of scanning electron microscopy (SEM) for observation of morphological structures that are difficult to observe with LM. The use of molecular tools, combined with morphological examinations, may assist taxonomists in addressing the suggestion of Moravec (2004) that a fundamental re-evaluation is necessary regarding the taxonomy and classification of dracunculoids. However, to date, only few molecular studies have been carried out on philometrid nematodes (Wu et al. 2005, Wijová et al. 2006, Quiazon et al. 2008).

The genus *Philometra* Costa, 1845 currently contains 112 reported species. These infect various organs, tissues and body cavities of a wide range of host fishes worldwide (Moravec 2006). To date, 16 *Philometra* species have been reported from Japan, namely *P. lateolabracis* (Yamaguti, 1935), *P. opsalichthydis* Yamaguti, 1935, *P. pinnicola* (Yamaguti, 1935), *P. parasiluri* Yamaguti, 1935, *P. scomberomori* (Yamaguti, 1935), *P. manangatuwo* Yamaguti, 1941, *P. inimici* Yamaguti, 1941, *P. sciaenae* Yamaguti, 1941, *P. sebastisci* Yamaguti, 1941, *P. sebastodis* Yamaguti, 1941, *P. cryptocentri* Yamaguti, 1961, *P. spari* Yamaguti, 1961, *P. plotosi* Moravec et Nagasawa, 1989, *P. ocularis* Moravec, Ogawa, Suzuki, Miyazaki et Donai, 2002, *P. madai* Quiazon, Yoshinaga et Ogawa, 2008, and *P. isaki* Quiazon, Yoshinaga et Ogawa, 2008. Of these species, seven (*P. lateolabracis*, *P. scomberomori*, *P. manangatuwo*, *P. sciaenae*, *P. sebastisci*, *P. madai* and *P. isaki*) have been reported from the gonads, while others from various body parts of their respective host fishes (Yamaguti 1935, 1941, 1961, Moravec and Nagasawa 1989, Moravec et al. 1998, 2002, Quiazon et al. 2008). Identification of these 112 Philometra species was based primarily on LM observations of the females, which can be easily detected due to their large size. However, males typically display more inter-specific variation than females. Despite this, very few males were collected due to their small size in most of the congeneric species.

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Even males of *P. lateolabracis*, a frequently reported gonad-infecting species, have not been described from the type host until very recently.

Philometra lateolabracis, which was mistakenly referred by Quiazon et al. (2008) as the type species of the genus *Philometra*, has been reported from a wide range of host fishes (Yamaguti 1935, Crisp and Klein 1973, Sakaguchi et al. 1987a, b, Sharples and Evans 1995a, b, Hesp et al. 2002, Moravec et al. 2003, Moravec and Genc 2004, Merella et al. 2004, Moravec and Justine 2005, Moravec 2006). Recently, Quiazon et al. (2008) conducted a detailed examination of male and female *P. lateolabracis* collected from its type host, *Lateolabrax japonicus* (Cuvier) (Japanese seaperch). They also collected two *Philometra* species previously identified as *P. lateolabracis* from *Pagrus major* (Temminck et Schlegel) (red seabream) and *Parapristipoma trilineatum* (Thunberg) (chicken grunt) and identified them as new species based on morphological and molecular differences from *P. lateolabracis* obtained from *L. japonicus*. Their study emphasized the need for describing males, detailed scanning electron microscopic observation and molecular examination for precise species identification of philometrid nematodes, in combination with the conventional light microscopic observation.

In this study, male and female philometrid nematodes infecting gonads of *Scomberomorus niphonius* (Cuvier) (Japanese Spanish mackerel), *Nemipterus virgatus* (Houttuyn) (golden threadfin bream) and *Pennahia argentata* (Houttuyn) (silver croaker) were collected. The nematode specimens were examined morphologically and molecularly to identify them at species level and to clarify their current taxonomic positions.

MATERIALS AND METHODS

Philometrid nematodes were isolated from the ovaries of *S. niphonius*, *N. virgatus* and *P. argentata*, collected near Awaji Island (Hyogo Prefecture), off Ichiki (Kagoshima Prefecture) and within Ariake Sound (Nagasaki Prefecture), respectively (Fig. 1). We examined fresh gonads of *N. virgatus* and *P. argentata*. The gonads of *S. niphonius* were fixed in 70% ethanol prior to examination. Female parasites were collected macroscopically from the gonads. For detection and collection of male parasites, gonads were pressed between two glass plates and examined under a stereomicroscope.

Male and female philometrids were fixed in 70% ethanol and cleared in glycerin for light microscopic examinations. After clearing, philometrids were mounted on slides. For long and coiled females, only the anterior and posterior portions were mounted on slides after measuring the total body length. Measurement and observation were performed using LM. All measurements were in millimetres. The general features of males, females and first-stage larvae, from the uterus of a fully gravid female philometrid, were drawn using a compound light microscope with a Nikon drawing tube attached. Females were categorised as gravid, subgravid and nongravid according to Quiazon et al. (2008). The new *Philometra* species, together with other species examined in this study, were deposited at the Meguro Parasitological Museum, Tokyo (M.P.M. Coll. Nos. 18859, 18861, 18863) and the Institute of Parasitology, Biology Centre of Academy of Science of the Czech Republic (BC ASCR), České Budějovice (Nos. N-916, N-917, N-703).

We used SEM to observe and measure the structures that could not be examined using LM. Prior to SEM examination, the 70% ethanol-fixed specimens were post-fixed in 1.25–1.5% glutaraldehyde, post-fixed in 1% osmium tetroxide and dehydrated through series of ascending ethanol concentrations. The samples were subjected to three changes of absolute butyl alcohol and freeze-dried. The freeze-dried samples were subsequently sputter-coated with gold and observed under a scanning electron microscope (SEM S-4000 Hitachi, Japan).

Subsamples of nematodes were fixed in 100% ethanol for molecular analysis. The genomic DNA of one male and three females, collected from each fish species, was extracted individually using a DNeasy™ Tissue Kit from Qiagen Inc. (protocol for animal tissues). The forward primer NC5f (5'-GTAGGTGAACCTGCGGAAGGATCATT-3') and reverse primer NC2r (5'-TTTAGTTTCTTTTCCTCCGCT-3'), designed by Zhu et al. (1998) for sequencing the ITS ribosomal DNA (rDNA) region (ITS1-5.8S rDNA-ITS2) of anisakid nematodes, were used for PCR amplification. PCR was performed using 1 µl of sample DNA as a template in a total volume of 20 µl, containing 0.6 µl forward and reverse primers, 14.1 µl double distilled water and 3.7 µl Taq mix (containing 0.1 µl TAKARA Ex Taq™ HS, 2 µl [10×] Ex Taq Buffer and 48 µl dNTP mixture). The DNA was initially denatured at 94°C for 4 min. Following this, 30 cycles of amplification were carried out using iCycler™ (BIO-RAD, Japan). Each cycle consisted of denaturing at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 30 sec and final extension at 72°C for 7 min.

Nucleotide bases were sequenced using a DNA automatic sequencer (ABI Prism® 310 Genetic Analyzer, Applied Biosystems, Japan) following purification of the ITS rDNA region. The sequenced DNA was individually checked for contamination by performing a BLAST search in NCBI (http://www.ncbi.nlm.nih.gov/). The boundaries between ITS1, 5.8S rDNA, ITS2, and 28S rDNA were determined manually by comparison with other reported nematodes in the GenBank database. Only the sequences encompassing the ITS2 region were used for analysis. The obtained sequences were aligned and percentage similarities (sequence identity matrix) were calculated using Clustal_W (Thompson et al. 1994) and Bioedit version 7.0.9.0 (Ibis Biosciences, Carlsbad, CA, USA).

Molecular comparisons were performed with other reported sequences of *Philometra* and *Philometroides* Yamaguti, 1935 in GenBank database (Table 1). Reported sequence of *Philometra fujimotoi* Furuyama, 1932 was not used because Margolis and Moravec (1987) transferred this species to the genus *Clavinema* Yamaguti, 1935, a change that was confirmed by Moravec (2006). *Philometroides pseudaspii* Moravec et Ergens, 1970 was among the valid species in the monograph of Moravec (2006); it was synonymized with *Philometroides ganzhounensis* Yu, 1998 whose ITS sequence was reported by Wu et al. (2005) in GenBank database. In contrast, *Philometroides carassii* (Ishii, 1931) was not listed in

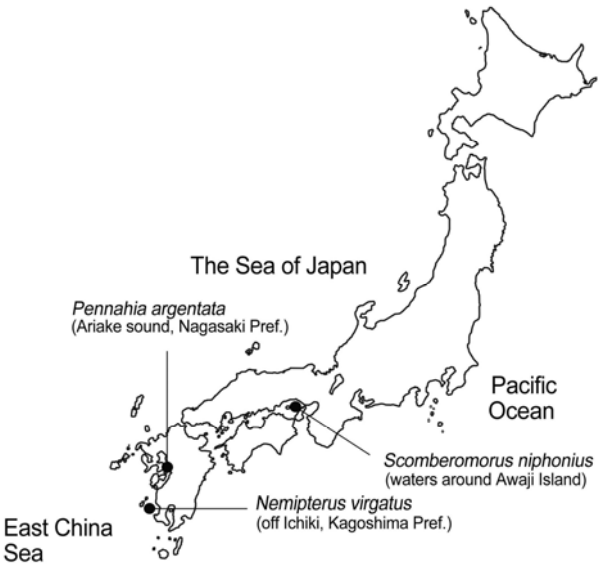

Fig. 1. Geographical locations of host fish collections in Japan.

the monograph of Moravec (2006) but its ITS sequence is available in GenBank database. Instead, *Philometra carassii* Ishii, 1931 was synonymized with the reported valid species *Philometroides sanguineus* (Rudolphi, 1819).

Due to technical problems during sequencing of the ITS1-5.8S region, only the data encompassing the beginning of the ITS2 and a few beginning sequences of the 28S rDNA were deposited in GenBank under the accession numbers EU443201, EU443202 and EU443203. Based on *p-distance* values in ITS2 region, the phylogenetic tree was inferred using neighbour-joining method (Saitou and Nei 1987) with the aid of MEGA 4.0 program (Tamura et al. 2007). Using non-parametric bootstrap analysis (Felsenstein 1985) with 1,000 replicates, the reliability of phylogenetic relationships was evaluated. The reliability was considered to be high if bootstrap values exceeded 70% of the replicates (Hillis and Bull 1993).

RESULTS

MORPHOLOGICAL STUDIES

Philometra sawara sp. n. Figs. 2, 3

Male (18 specimens, holotype and paratypes, collected in August 2003, January 2004 and January 2005): Body filiform; length 2.44–3.38; cuticle smooth; transparent to whitish-cream body colouration when alive; body widest near mid-section (0.045–0.070), tapering gradually towards anterior section before broadening leading to bulbous terminal structure near round anterior end; overall oesophagus length 0.252–0.460, enlarged (bulb formation) near anterior end; distinct oesophageal gland with large rounded nucleus, 0.008–0.016 in diameter, visible near mid-section; anterior section of oesophagus 0.065–0.220 in length; posterior section overlapped by oesophageal gland 0.16–0.24 in length; distance from anterior end to oesophageal gland nucleus and nerve ring 0.169–0.283 and 0.084, respectively; ventriculus length and width 0.02 and 0.03, respectively; testis extended posteriorly to base of spicules, with white spots visible along each testis; spicules narrow, needle-like, and of unequal length; longer spicule 0.074–0.135 in length; shorter spicule 0.071–0.131 in length; length ratio of spicules 1:1.03–1.05; gubernaculum narrow, 0.040–0.076 long, with proximal end bent dorsally and with lamellate-like structures; length ratio of longer spicule and gubernaculum 1:1.34–1.88; posterior end of body rounded with two large lobes on both sides of spicules and gubernaculum; each lobe subdivided into two smaller lobes nearly equal in size, with hardly visible papillae; no phasmid outlets observed.

Gravid female (10 specimens, allotype and paratypes, collected in April, May and August 2003, and between January and February 2004): Body filiform; length 68–193; cuticle smooth; slight yellowish brown to reddish body colouration; intestine light to dark-brown in colour when alive; body widest near mid-section (0.71–1.70), tapering gradually towards posterior end; anterior end of body broad and rounded; cephalic end dome-shaped with four submedian pairs of cephalic papillae arranged in outer ring, and four single papillae forming inner ring; paired cephalic papillae slightly rounded and elongated; mouth opening 0.025 in diameter, with pair of amphids on both sides; overall oesophagus length 0.76–1.145, broad and highly enlarged near mouth forming very distinct bulb (0.110–0.165 long and 0.116–0.180 wide); narrowest width of oesophagus around nerve ring (0.058–0.100 in diameter); oesophageal gland prominent, extending anteriorly to nerve ring and posteriorly to ventriculus; large round nucleus, 0.015–0.048 in diameter, located near mid-section of oesophageal gland; anterior section of oesophagus 0.202–0.325 in length; posterior section partially overlapped by oesophageal gland 0.545–0.772 in length; distance from anterior end to oesophageal gland nucleus and nerve ring 0.468–0.705 and 0.170–0.265, respectively; ventriculus well developed, 0.095–0.150 in length and 0.082–0.130 in width; two long ovaries situated near anterior and posterior ends of body; anterior ovary extending near mid-section of oesophagus; posterior ovary extending nearly to body end; uterus occupying majority of body space and filled with developing embryos and first-stage larvae; posterior end of body rounded; intestine relatively narrow, straight and atrophied near posterior end, forming ligament attached ventrally to body wall, anterior to posterior end; lateral papilla-like caudal projections hardly visible; no vagina or vulva observed.

Subgravid female (2 specimens collected in February 2004): Body length 46.7–64.5.

First-stage larva (10 larvae from females collected in August 2003): Body length 0.508–0.542; width 0.014–0.018; oesophagus, intestine, and tail comprising 26–31%, 40–45%, and 28–31% of the total length, respectively.

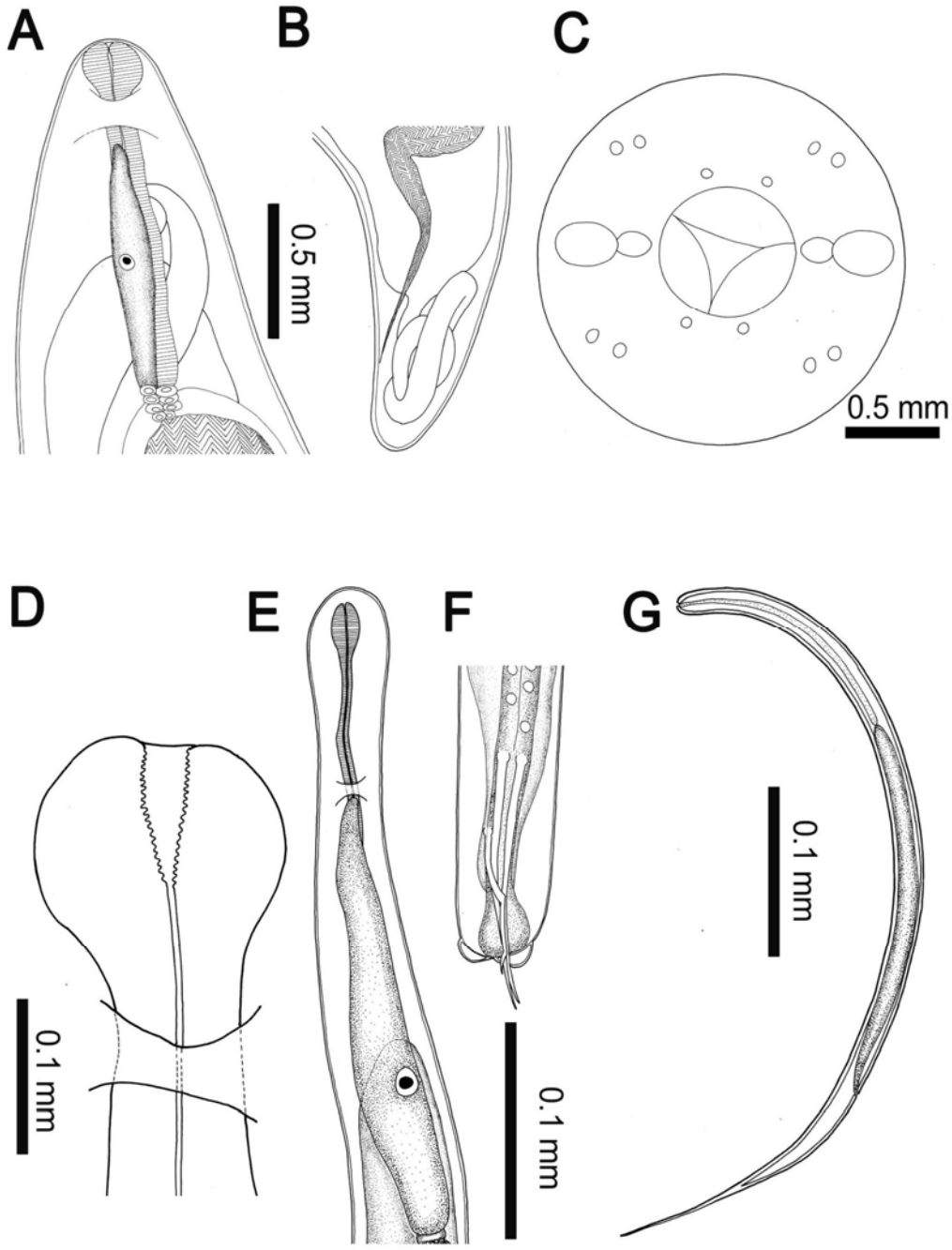

Fig. 2. *Philometra sawara* sp. n. **A, B** – anterior and posterior end of female; **C** – cephalic end of female, apical view; **D** – highly inflated anterior end of the oesophagus in female; **E, F** – anterior and posterior end of male; **G** – first-stage larva.

T y p e h o s t : *Scomberomorus niphonius* (Cuvier) (Perciformes: Scombridae); FishBase name: Japanese Spanish mackerel; Japanese name: sawara.

H o s t ' s b o d y s i z e : Fork length, 400–520 mm; body weight, 475–975 g.

S i t e o f i n f e c t i o n : Ovary.

D a t e o f c o l l e c t i o n : April, May and August 2003; January and February 2004; January 2005.

P r e v a l e n c e : 71% (43 fish infected out of 61 fish examined).

I n t e n s i t y : Male parasites, 1–6 per fish; female parasites, 1–11 per fish.

T y p e l o c a l i t y : Northern (134˚N, 35˚E) and southern (136˚N, 34˚E) part of Awaji Island, Hyogo Prefecture, Seto Inland Sea, Japan.

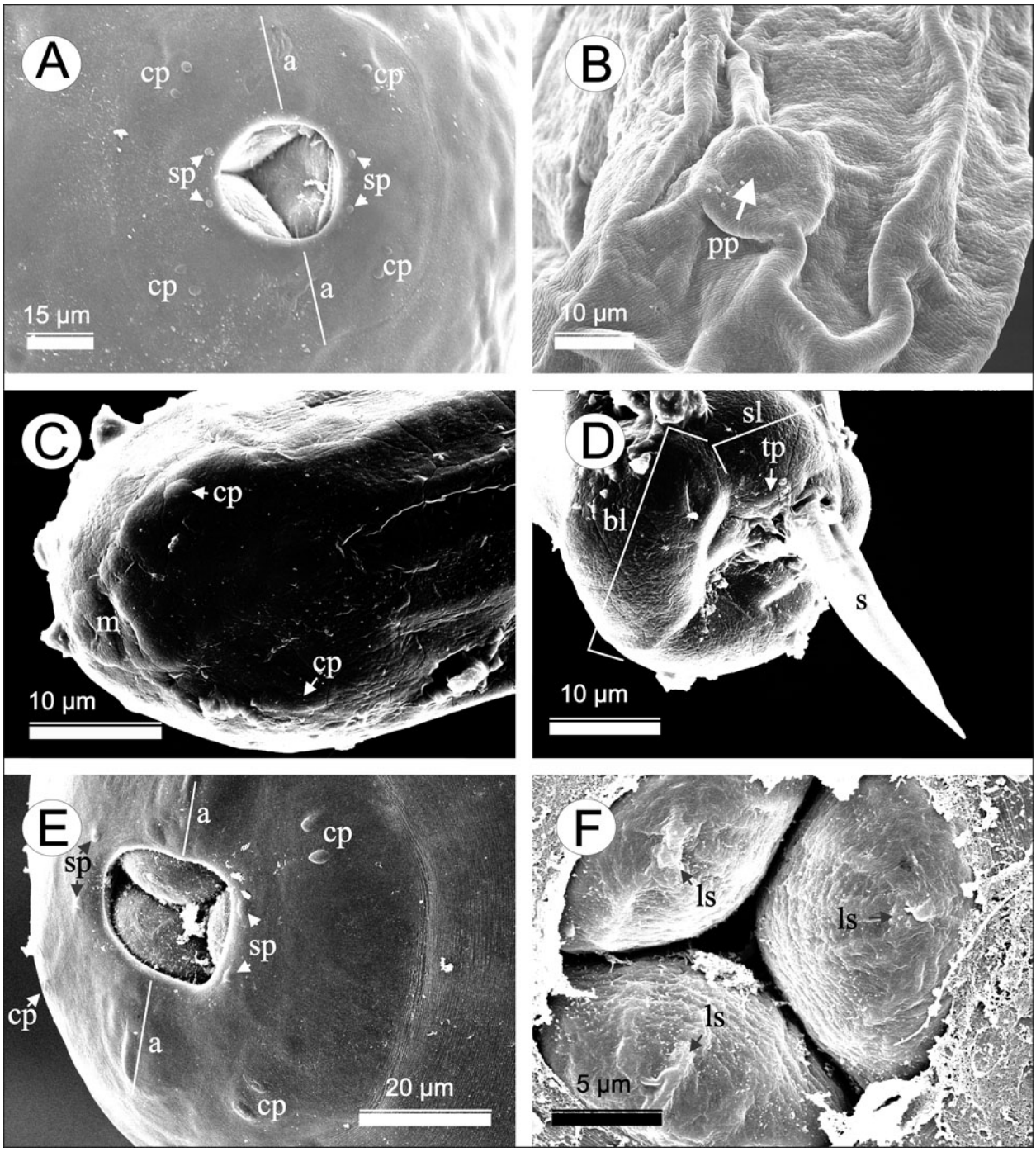

Fig. 3. Scanning electron micrographs of *Philometra sawara* sp. n. (A, B) and *Philometra nemipteri* Luo, 2001 (C–F). **A** – cephalic end of female *P. sawara*, apical view; **B** – closer view of the lateral papilla-like caudal projection at the caudal end; **C** – cephalic end of male *P. nemipteri*; **D** – posterior end of male *P. nemipteri*; **E** – cephalic end of female *P. nemipteri*, subapical view; **F** – closer view of anterior end of the oesophagus of female *P. nemipteri* showing the presence of the protruding lobular structure at the anterior tip in each oesophageal lobe. *Abbreviations*: a – amphids; bl – bigger-sized and U-shaped lobular mound; cp – paired cephalic papillae of outer ring; ls – protruding lobular structure at the anterior tip in each oesophageal lobe; m – mouth; s – spicules; pp – papilla-like caudal projections; sl – smaller-sized lobe; sp – single papillae of inner ring; tp – tail papillae.

D e p o s i t i o n o f s p e c i m e n s : Male holotype, allotype and paratypes deposited in the Meguro Parasitological Museum, Tokyo (M.P.M. Coll. No. 18859). Paratypes also deposited in the Institute of Parasitology, BC ASCR, České Budějovice (N-916).

E t y m o l o g y : The species name relates to the Japanese name of the fish host, i.e., sawara.

Comments. It is possible that the parasite described here, collected from the ovary of *S. niphonius*, is identical with the originally described *Sanguinofilaria scomberomori* Yamaguti, 1935, collected from *S. sinensis* (Lacépède) caught in the Pacific Ocean near Japan. Both host fishes belong to the same genus, *Scomberomorus* Lacépède. *Sanguinofilaria* was synonymized by Yamaguti (1941) with *Philometra*, now the current genus for this species. However, a detailed comparison between the two philometrids was not possible because males of *P. scomberomori* had not been collected. Also, the original description of female *P. scomberomori* by Yamaguti (1935) was relatively short and museum specimens of *P. scomberomori*, deposited by Yamaguti (1935), were not available. A comparison with the short descriptions made by Yamaguti (1935) revealed some morphological differences. These include the wider body (0.9) and narrower bulb (0.10–0.11) at the inflated anterior end of the oesophagus in gravid female of *P. sawara*. Also, first-stage larvae of *P. sawara* were longer than those of *P. scomberomori* (0.40). Comparisons with other *Philometra* species reported from the gonads of other host fishes of the same family Scombridae also indicated an independent species (Moravec 2006). *Philometra globiceps* (Rudolphi, 1819), the type species of the genus *Philometra*, has been reported from *Scomberomorus maculatus* (Mitchill), but this was questioned by Moravec (2006). Morphologically, *P. globiceps* from its type host *Uranoscopus scaber* Linnaeus has longer body (1.67–6.16) and longer equal spicules (0.137–0.156) in males, shorter (60) and narrower (0.6) body, and smaller round mouth (approximately 0.4–0.45) in females, and longer first-stage larvae (0.61) compared to *P. sawara. Philometra katsuwoni* Petter et Baudin-Laurencin, 1986 reported from *Katsuwonus pelamis* (Linnaeus) has relatively longer (9.5–12.0) and wider (0.09–0.11) body, longer oesophagus (0.74–1.65), greater distance of nerve ring to anterior end (0.2–0.25), different spicule length (right, 1.75–2.08; left, 0.065–0.95) and longer gubernaculum (0.130–0.145) compared with *P. sawara. Philometra macroandri* (Shchepkina, 1978), reported from *Thunnus alalunga* (Bonnaterre) (type host) and *Thunnus albacores* (Bonnaterre) has also relatively longer (11.3–19.78) and wider (0.252) body, longer oesophagus (0.536–1.00), greater distance of nerve ring to anterior end (0.23) and shorter gubernaculum (0.022–0.040) compared to *P. sawara.*

Rasheed (1963) considered *P. scomberomori* a junior synonym of *P. lateolabracis.* Given that conspecific males of both *P. scomberomori* and *P. lateolabracis* were unknown at that time to confirm this, both were treated by Moravec (2006) as independent species. With the repeated reporting of female *P. lateolabracis* from different fish families, the possibility that *P. sawara* is also a junior synonym of *P. lateolabracis* cannot be excluded. However, morphological comparisons carried out with the newly described male and redescribed female *P. lateolabracis* by Quiazon et al. (2008) revealed that *P. sawara* and *P. lateolabracis* are independent species. Major differences was observed on the bigger round mouth and highly inflated anterior end of the oesophagus in female *P. sawara* compared to the small triangular mouth and lightly inflated anterior end of the oesophagus in female *P. lateolabracis.* In males, major difference was observed in the presence of white spots aligned along the testis in 8 out of 18 specimens examined. This feature might be due to the maturity stage of the males, but this was never observed by Quiazon et al. (2008) in 20 male *P. lateolabracis*.

On the other hand, comparisons carried out with other *Philometra* species identified in Japan (Quiazon et al. 2008) also revealed independent species. Female *P. isaki* has small triangular mouth and slightly distinct bulb at the anterior end of oesophagus, while male has no white spots along the testis. Female *P. madai* has highly distinct bulb at the anterior end of oesophagus, similar with that of *P. sawara*, but different with regard to the relatively narrow oesophagus. Lastly, male *P. madai* has relatively longer body (3.92–5.94) and U-shaped lobular mound at the posterior end, while male *P. sawara* has shorter body length and equal-sized posterior lobes.

In view of these differences, we are confident that the specimens isolated from *S. niphonius* represent a new species, unless sufficient morphological information (as well as molecular data), from both male and female *P. scomberomori* from *S. sinensis*, become available.

Philometra nemipteri Luo, 2001 Figs. 3, 4

Male (11 specimens collected in September 2005): Body filiform; length 2.94–4.02; cuticle smooth; transparent to whitish-cream body colouration when alive; body widest near mid-section (0.068–0.092), tapering gradually towards anterior section before broadening leading to a bulbous terminal structure near rounded anterior end; cephalic end dome-shaped with four submedian pairs of cephalic papillae arranged in outer ring, and four single papillae forming inner ring; mouth opening relatively small (0.001–0.002 in diameter) with pair of amphids situated laterally; overall oesophagus length 0.423–0.478, enlarged (bulb formation) near anterior end; distinct oesophageal gland with large rounded nucleus, 0.007–0.012 in diameter, visible near mid-section; anterior section of oesophagus 0.131–0.205 in length; posterior section overlapped by oesophageal gland 0.225–0.330 in length; distance from anterior end

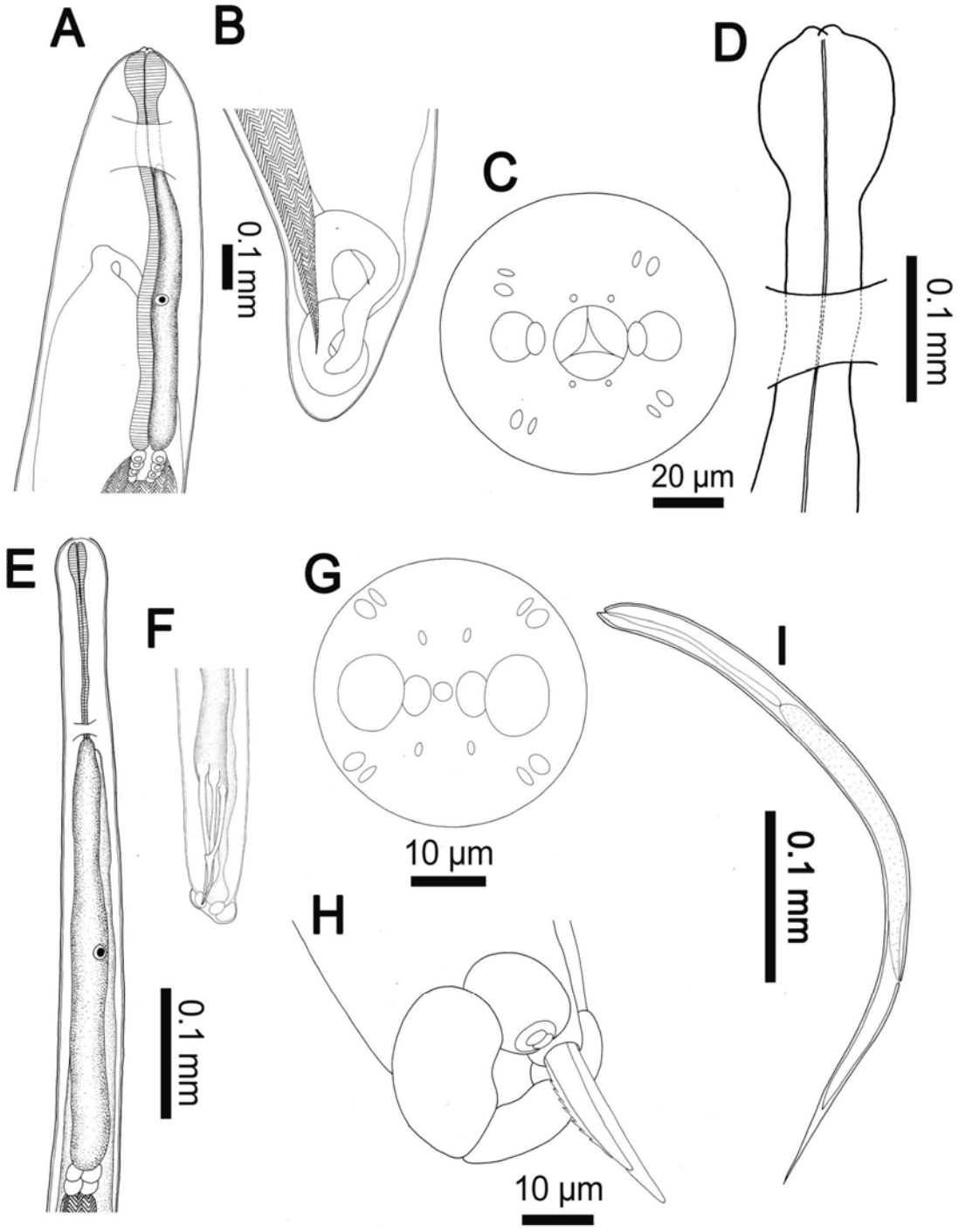

Fig. 4. *Philometra nemipteri* Luo, 2001. **A, B** – anterior and posterior end of female; **C** – cephalic end of female, apical view; **D** – moderately inflated anterior end of oesophagus with protruding small lobular structure at the anterior tip in each oesophageal lobe in female; **E, F** – anterior and posterior end of male; **G** – cephalic end of male, apical view; **H** – closer view of posterior end of male showing two smaller-sized lobes attached to a bigger-sized and U-shaped lobular mound; **I** – first-stage larva.

to oesophageal gland nucleus and nerve ring 0.308–0.340 and 0.15–0.19, respectively; ventriculus length and width 0.015–0.023 and 0.018–0.027, respectively; testis extended posteriorly to base of spicules; spicules narrow, needle-like, and of unequal length with passage canal for sperm cells located in central section of spicules; longer spicule 0.093–0.126 in length; shorter spicule 0.085–0.113 in length; length ratio of spicules 1:1.02–1.21; gubernaculum narrow (0.073–0.101 in length), with proximal end bent dorsally and with lamellate-like structures; length ratio of longer spicule to gubernaculum 1:1.08–1.66; posterior end of body

rounded with two smaller posterior lobes connected by a broad, U-shaped, lobular mound on both sides of spicules and gubernaculum; caudal papillae observed on posterior end of each pair of smaller lobes; no phasmid outlets observed.

Gravid female (15 specimens collected in September 2005): Body filiform; length 23–85; cuticle smooth; yellowish brown to reddish body colouration; intestine light to dark-brown in colour when alive; body widest near mid-section (0.28–0.74), tapering gradually towards posterior end; anterior end of body rounded; cephalic end dome-shaped with four submedian pairs of cephalic papillae arranged in outer ring, and four single papillae forming inner ring; paired cephalic papillae slightly rounded and elongated; mouth opening 0.020–0.025 in diameter, with pair of amphids on both sides; overall oesophagus length 0.655–1.025, highly enlarged near mouth forming distinct bulb (0.085–0.120 long and 0.078–0.110 wide), with protruding lobular structures on anterior tip of each oesophageal lobe; narrowest width of oesophagus around nerve ring (0.035–0.060 in diameter); oesophageal gland prominent, extending anteriorly to nerve ring and posteriorly to ventriculus; large round nucleus, 0.018–0.028 in diameter, located near mid-section of oesophageal gland; anterior section of oesophagus 0.175–0.250 in length; posterior section partially overlapped by oesophageal gland 0.477–0.800 in length; distance from anterior end to oesophageal gland nucleus and nerve ring 0.418–0.627 and 0.175–0.245, respectively; ventriculus well developed, 0.040–0.085 in length and 0.060–0.088 in width; two long ovaries situated near anterior and posterior ends of body; anterior ovary extending near mid-section of oesophagus; posterior ovary extending nearly to body end; uterus occupying majority of body space and filled with developing embryos and first-stage larvae; posterior end of body rounded; intestine relatively narrow, straight, and atrophied near posterior end forming a ligament attached ventrally to body wall, anterior to posterior end; no vagina or vulva observed.

First-stage larva (10 larvae from females collected in September 2005): Body length 0.421–0.488; width 0.016–0.018; oesophagus, intestine, and tail comprising 26–34%, 37–45%, and 27–32% of total length, respectively.

Host: *Nemipterus virgatus* (Houttuyn) (Perciformes: Nemipteridae); FishBase name: golden threadfin bream; Japanese name: itoyoridai.

Host's body size: Total length, 246–412 mm; body weight, 96–409 g.

Site of infection: Gonads.

Date of collection: September 2005.

Prevalence: 87% (26 fish infected out of 30 fish examined).

Intensity: Male parasites, 1–2 per fish; female parasites, 1–23 per fish.

Locality: Off Ichiki, Kagoshima Prefecture, East China Sea, Japan (130°N, 31°E).

Deposition of specimens: Meguro Parasitological Museum, Tokyo (M.P.M. Coll. No. 18861) and the Institute of Parasitology, BC ASCR, České Budějovice (N-917).

Comments. Luo (2001) first reported and described only female *P. nemipteri* from *N. virgatus* collected in Taiwan Straight (Minnan-Taiwan Bank Fishing Ground). Moravec (2006) concluded that this species was *species inquirenda* because of an inadequate description. In the present study, male and female philometrids were found in the gonads of the same host species collected in Kagoshima Prefecture, Japan (East China Sea). Although morphological description by Luo (2001) was insufficient, our female specimens have similar morphological features to those of *P. nemipteri* specimens described by Luo (2001) (i.e., similar in total body length, maximum body width, oesophagus length, structure on the anterior oesophagus, and structures on the anterior and posterior ends of the body). Given that both philometrids appear to be morphologically similar and originated from the same host species, the current philometrid specimen was identified as *P. nemipteri*. Moravec (2006) mentioned the similarity of *P. nemipteri* and *P. lateolabracis* based on the morphology of females. However, both male and female *P. nemipteri* examined in the present study, and those of *P. lateolabracis* reported by Quiazon et al. (2008), were distinguishable morphologically. Male *P. nemipteri* has longer and wider body, longer oesophagus, more distinct inflation (bulb formation) at the anterior end of the oesophagus, greater distance between the oesophageal gland nucleus and the anterior end, and have a broad U-shaped lobular mound, connecting the two posterior lobes on both sides of the spicules and gubernaculum, when compared to male *P. lateolabracis* (Quiazon et al. 2008). Similarly, female *P. nemipteri* have a round-shaped mouth, a highly swollen anterior oesophagus forming distinct bulb, protruding lobular structures at the anterior tip in each of the three oesophageal lobes, and a shorter body in fully gravid females compared to female *P. lateolabracis*, which have triangular mouth, slight inflation at the anterior end of the oesophagus and a longer body in fully gravid females (Quiazon et al. 2008).

Philometra sciaenae Yamaguti, 1941 Figs. 5, 6

Male (21 specimens collected in June and September 2004): Body filiform; length 1.46–2.62; cuticle smooth; transparent to whitish-cream body colouration when alive; body widest near mid-section (0.040–0.076), tapering gradually towards anterior section without formation of bulbous terminal structure near round anterior end; cephalic end dome-shaped with four submedian pairs of cephalic papillae arranged in outer ring, and four single papillae forming inner ring; mouth

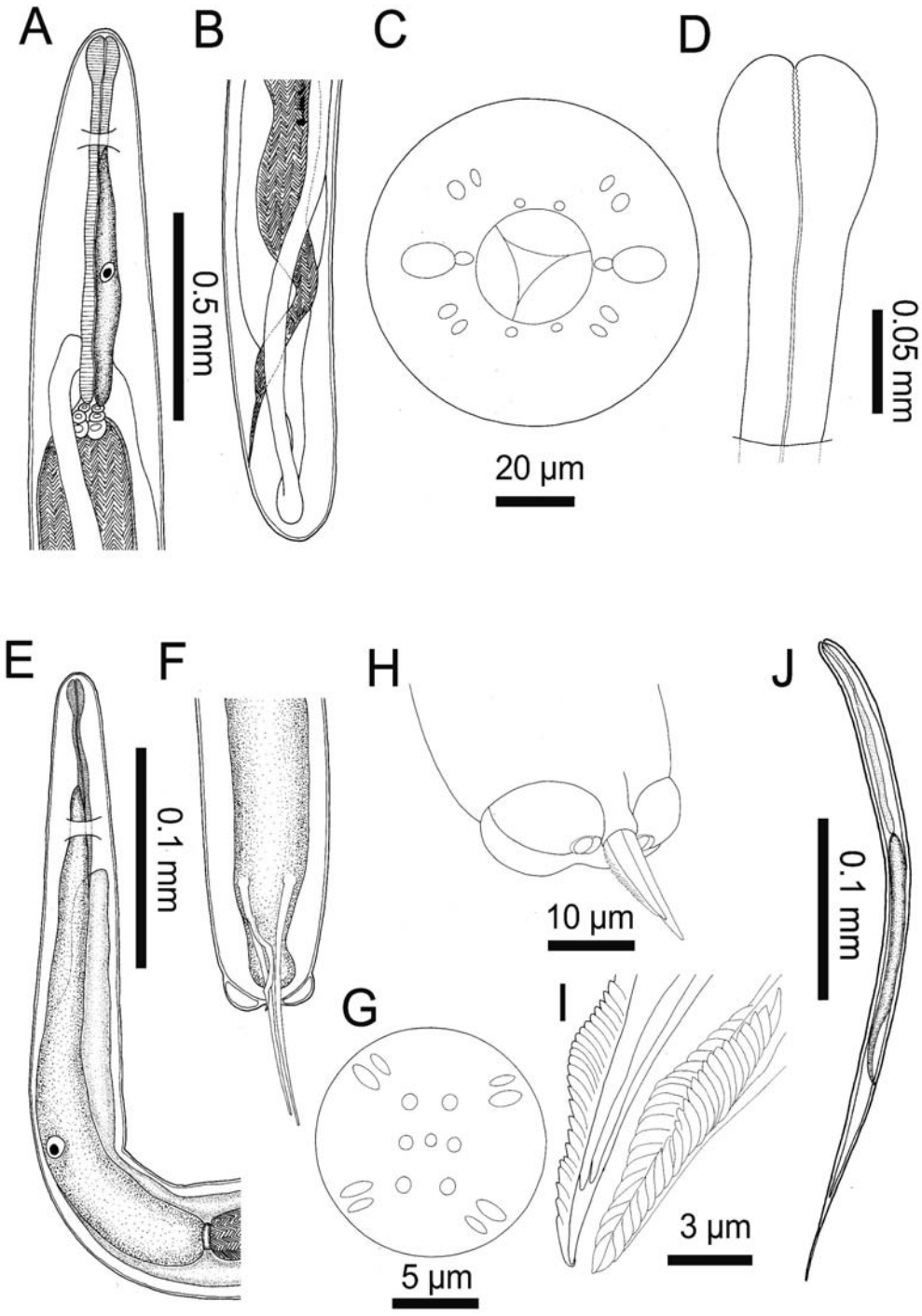

Fig. 5. *Philometra sciaenae* Yamaguti, 1941. **A, B** – anterior and posterior end of female; **C** – cephalic end of female, apical view; **D** – moderately inflated anterior end of oesophagus in female; **E, F** – anterior and posterior end of male; **G** – cephalic end of male, apical view; **H** – closer view of posterior end of male showing equal-sized subdivided lobes, tail papillae, spicules and gubernaculum; **I** – closer view of spicules and gubernaculum, top and sub-ventral view; **J** – first-stage larva.

opening relatively small (0.001 in diameter) with pair of amphids situated laterally; overall oesophagus length 0.245–0.390, enlarged (bulb formation) near anterior end; oesophageal gland very broad with large rounded nucleus, 0.008–0.011 in diameter, visible near mid-section; anterior section of oesophagus 0.033–0.140 in length; posterior section overlapped by oesophageal gland 0.16–0.30 in length; distance from anterior end to oesophageal gland nucleus and nerve ring 0.140–0.255 and 0.032–0.070, respectively; ventriculus present but

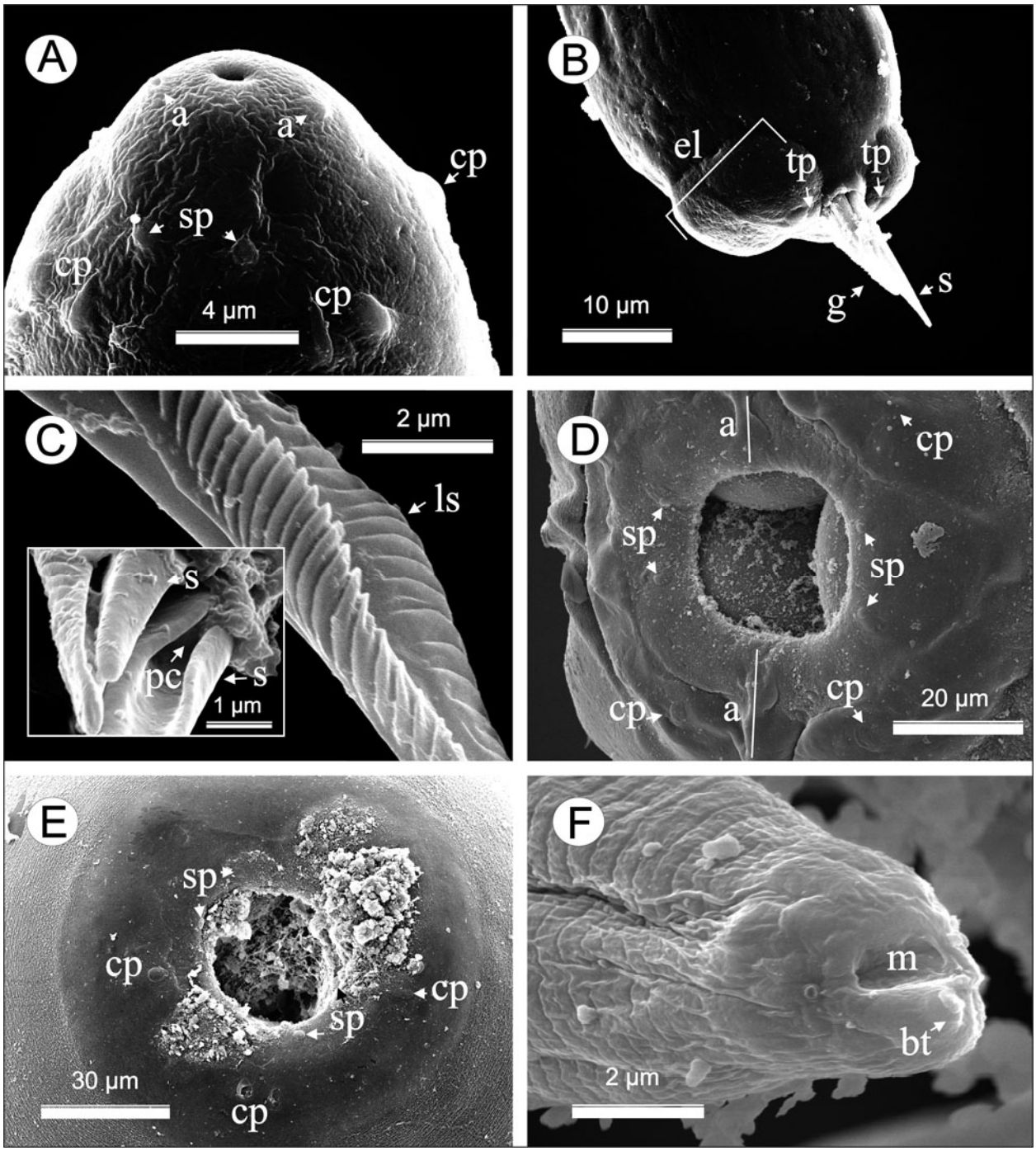

Fig. 6. Scanning electron micrographs of *Philometra sciaenae* Yamaguti, 1941. **A** – cephalic end of male; **B** – posterior end of male; **C** – closer view of spicules and lamellate-like structures of the gubernaculum (inset – closer view of spicules showing the duct for releasing sperm cells); **D** – cephalic end of female; **E** – (another specimen) cephalic end of female; **F** – anterior end of first-stage larva. *Abbreviations*: a – amphids; bt – boring tooth; cp – paired cephalic papillae of outer ring; el – equal-sized lobes; g – gubernaculum; ls – lamellate-like structure of the gubernaculum; m – mouth; pc – passageway canal for sperm cells; s – spicules; sp – single papillae of inner ring; tp – tail papillae.

barely visible; testis extended posteriorly to base of spicules; spicules narrow, needle-like, and of unequal length with passage canal for sperm cells located in central section of spicules; longer spicule 0.098–0.138 in length; shorter spicule 0.096–0.135 in length; length ratio of spicules 1:1.02–1.06; gubernaculum narrow

(0.045–0.074 in length), with proximal end bent dorsally and with lamellate-like structures; length ratio of longer spicule and gubernaculum 1:1.23–2.08; posterior end of body rounded with two large lobes on both sides of spicules and gubernaculum; each lobe subdivided into two smaller lobes of nearly equal size; caudal papillae observed on posterior end of each pair of large lobes; no phasmid outlets observed.

Gravid female (10 specimens collected in September 2004): Body filiform; length 44–104; cuticle smooth; yellowish brown to reddish body colouration; intestine brownish to dark brown in colour when alive; body widest near mid-section (0.40–0.65), tapering gradually towards posterior end; anterior end of body rounded; cephalic end dome-shaped with four submedian pairs of cephalic papillae arranged in outer ring, and four single papillae forming inner ring; paired cephalic papillae slightly rounded and elongated; mouth opening 0.025 in diameter, with pair of amphids on both sides; overall oesophagus length 0.760–0.945, enlarged near mouth forming distinct bulb (0.088–0.115 long and 0.072–0.088 wide); narrowest width of oesophagus around nerve ring (0.038–0.055 in diameter); oesophageal gland prominent, extending anteriorly to nerve ring and posteriorly to ventriculus; large round nucleus, 0.035 in diameter, located near mid-section of oesophageal gland; anterior section of oesophagus 0.210–0.228 in length; posterior section partially overlapped by oesophageal gland 0.532–0.735 in length; distance from anterior end to oesophageal gland nucleus and nerve ring 0.480 and 0.220–0.270, respectively; ventriculus well developed, 0.070–0.080 in length and 0.075–0.080 in width; two long ovaries situated near anterior and posterior ends of body; anterior ovary extending near mid-section of oesophagus; posterior ovary extending nearly to body end; uterus occupying majority of body space and filled with developing embryos and first-stage larvae; posterior end of body rounded; intestine relatively narrow, straight and atrophied near posterior end, forming a ligament attached ventrally to body wall, anterior to posterior end; no vagina or vulva observed.

Subgravid female (5 specimens collected in June and September 2004): Body length 11.38–28.82.

Nongravid female (13 specimens collected in June and September 2004): Body length 3.51–10.21.

First-stage larva (10 larvae from females collected in September 2004): Body length 0.320–0.413; width 0.014–0.016; oesophagus, intestine, and tail comprising 30–33%, 37–42%, and 27–31% of total body length, respectively.

Host: *Pennahia argentata* (Houttuyn) (Perciformes: Sciaenidae); FishBase name: silver croaker; Japanese name: shiroguchi.

Host's body size: Total length, 100–300 mm; body weight, 17–317 g.

Site of infection: Gonads.

Date of collection: June and September 2004.

Prevalence: 55% (126 fish infected out of 230 fish examined).

Intensity: Male parasites, 1–13 per fish; female parasites, 1–63 per fish.

Locality: Off Shimabara, Nagasaki Prefecture, Ariake sound, Japan (130°N, 33°E).

Deposition of specimens: Meguro Parasitological Museum, Tokyo (M.P.M. Coll. No. 18863) and the Institute of Parasitology, BC ASCR, České Budějovice (N-703).

Comments. Yamaguti (1941) originally described *P. sciaenae* from the ovary of *Pennahia argentata* (formerly *Sciaena schlegeli* and *Argyrosomus argentatus*) collected from Hamajima, Mie Prefecture, Japan. This species was considered a junior synonym of *P. lateolabracis* by Rasheed (1963). However, Moravec et al. (1998) collected and described two males (one complete and one fragmented body) for the first time, together with 17 females, from *P. argentata* (= *A. argentatus*), collected in the East China Sea, off Shimabara, Nagasaki Prefecture, Japan. Moravec (2006) treated *P. sciaenae* as a valid species in his monograph on dracunculoids.

A comparison of the present specimens with those of Moravec et al. (1998) revealed generally similar morphological features, with two minor differences. The current study found an inflation in the anterior portion of the oesophagus and nearly equal spicules (spicule ratio of 1:1.02–1:1.06) in all male specimens. Based on the identity and locality of the host species and general morphology of *P. sciaenae*, as described by Moravec et al. (1998), the current specimens were identified as *P. sciaenae*. The current description, from 21 whole male specimens, provides additional morphological information.

A comparison of the present male and female specimens with male and female *P. lateolabracis*, described by Quiazon et al. (2008), suggests that these parasites are entirely different species. Female *P. sciaenae* are shorter, have a longer oesophagus in relation to total body length and a narrower oesophagus at the nerve ring portion than *P. lateolabracis.* In the case of male *P. lateolabracis*, major difference is observed at the region of the anterior section, which gradually tapers towards the anterior end, then gradually broadens forming a distinct bulbous anterior extremity. In contrast, the anterior section of *P. sciaenae* gradually tapers towards the anterior end without forming any bulbous anterior extremity. Our results confirm the conclusion of Moravec et al. (1998) that *P. sciaenae* is independent from *P. lateolabracis* and should be recognized as a valid species.

Table 1. Estimates of evolutionary divergence in the ITS2 region of rDNA showing the percentage similarities[a], number of base differences[b] [in square brackets] and *p-distance* values[b] (in parentheses) between the current *Philometra* specimens and previously reported sequences of *Philometra* and *Philometroides* in GenBank database.

Species	No. bp	1	2	3	4	5	6	7	8	9	10	GenBank Acc. No.	Author
1. *Philometra sawara* [c]	479	ID										EU443203	present study
2. *Philometra nemipteri* [d]	425	79.3 [29] (0.070)	ID									EU443201	present study
3. *Philometra sciaenae* [e]	497	77.5 [53] (0.116)	71.6 [43] (0.105)	ID								EU443202	present study
4. *Philometra lateolabracis* [f]	489	71.5 [68] (0.154)	63.7 [53] (0.137)	73.4 [49] (0.109)	ID							EF203081	Quiazon et al. 2008
5. *Philometra madai* [g]	499	85.9 [36] (0.076)	74.1 [42] (0.101)	76.9 [66] (0.140)	70.5 [83] (0.181)	ID						EF203082	Quiazon et al. 2008
6. *Philometra clavaeceps* [h]	549	39.2 [199] (0.445)	36.2 [173] (0.439)	41.2 [207] (0.454)	40.4 [202] (0.449)	41.2 [215] (0.461)	ID					DQ076696	Wu et al. 2005
7. *Philometroides fulvidraconi* [i]	471	38.0 [173] (0.436)	35.6 [148] (0.418)	38.9 [173] (0.430)	38.0 [172] (0.439)	40.1 [181] (0.438)	72.0 [53] (0.116)	ID				DQ076694	Wu et al. 2005
8. *Philometroides ganzhounensis* [j]	551	38.8 [197] (0.447)	36.2 [169] (0.434)	40.5 [203] (0.453)	38.4 [199] (0.455)	40.9 [213] (0.462)	85.7 [15] (0.029)	72.8 [54] (0.117)	ID			DQ076691	Wu et al. 2005
9. *Philometroides cyprini* [k]	346	33.8 [166] (0.542)	34.9 [140] (0.513)	31.6 [155] (0.513)	32.6 [153] (0.543)	33.2 [173] (0.546)	27.3 [207] (0.614)	26.8 [181] (0.607)	27.5 [207] (0.611)	ID		DQ076697	Wu et al. 2005
10. *Philometroides carassii* [l]	407	43.5 [159] (0.412)	42.7 [144] (0.400)	40.4 [150] (0.380)	41.3 [149] (0.391)	42.9 [150] (0.385)	34.5 [172] (0.434)	36.8 [157] (0.435)	34.8 [177] (0.448)	52.2 [155] (0.556)	ID	DQ076693	Wu et al. 2005

[a] Sequence identity matrix computed using Bioedit version 7.0.9.0.
[b] All results are based on the pairwise analysis using MEGA4. All positions containing gaps and missing data were eliminated from the dataset (pairwise deletion option).
[c] Host: *Scomberomorus niphonius*; Locality: Awaji Island, Japan.
[d] Host: *Nemipterus virgatus*; Locality: East China Sea, Japan.
[e] Host: *Pennahia argentata* (=*Argyrosomus argentatus*); Locality: The Sea of Ariake, Japan.
[f] Host: *Lateolabrax japonicus*; Locality: Tokyo Bay, Japan.
[g] Host: *Pagrus major*; Locality: Seto Inland Sea, Japan.
[h] Host: *Chanodichthys erythropterus* (syn. *Cultrichthys erythropterus* as reported by Wu et al. 2005); Locality: Liangzi Lake, Hubei, China.
[i] Host: *Pelteobagrus fulvidraco*; Locality: Jiangkou reservoir, Jiangxi, China.
[j] Host: *Hemibarbus maculatus*; Locality: Doushui reservoir, Jiangxi, China; Note: *Philometroides ganzhounensis* and *Philometroides buirnurensis* Luo, Chen, Fang et Wang, 2004 were synonymized with *Philometroides pseudaspii* by Moravec (2006).
[k] Host: *Cyprinus carpio*; Locality: Baoan Lake, Hubei, China; Syn.: *Filaria cyprinid* Ishii, 1931, *Philometra lusii* Visman, 1962, *Philometra schikhobalowae* Belous, 1965; *Philometroides lusiana* Vismanis, 1966.
[l] Host: *Carassius auratus*; Locality: Tangxum Lake, Hubei, China; Note: This species (*Philometroides ganzhounensis*) is considered a synonym of *Philometroides sanguineus* (see Moravec 2006).

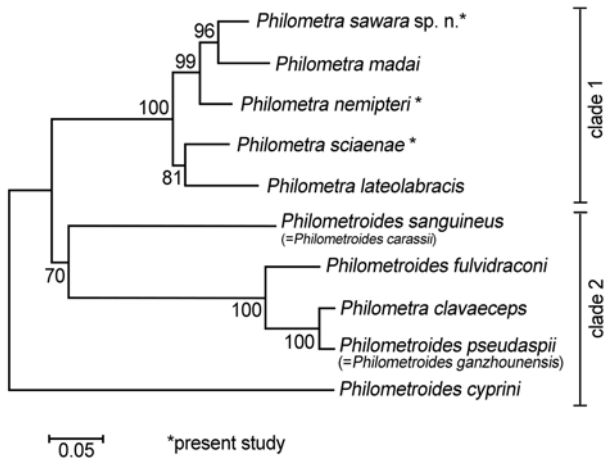

Fig. 7. Neighbour-joining (NJ) tree inferred from *p-distance* values based on the ITS2 SSU rDNA showing the genetic relationship among presently and previously studied *Philometra* species. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option).

MOLECULAR STUDIES

Philometra sciaenae, *P. sawara* and *P. nemipteri* had 497, 479 and 425 nucleotide bases, respectively, in the ITS2 region. No intraspecies variations were observed among four representative specimens for each species. *Philometra* and *Philometroides* species were divided into two major clades in the neighbour-joining tree calculated based on *p-distance* values (Fig. 7). One clade (clade 1) included five *Philometra* species, namely *P. sciaenae*, *P. sawara*, *P. nemipteri*, *P. lateolabracis*, and *P. madai*. The second clade (clade 2) included four *Philometroides* species (i.e., *Philometroides fulvidraconi* Yu, Wu et Wang, 1993, *Philometroides pseudaspii* (= *Philometroides ganzhounensis*), *Philometroides cyprini* (Ishii, 1931) and *Philometroides sanguineus* (= *Philometroides carassii*)) and one *Philometra* species (i.e., *P. clavaeceps* Dogiel et Akhmerov, 1959). As shown in Table 1, the interspecies similarity (similarity matrix) between philometrid species in clade 1 was 63.7–85.9%, with bootstrap probabilities above 81%. The number of different bases among the philometrid species in clade 1 was 29–83 bases. Our results indicate that the five species of *Philometra*, molecularly examined in Japan, were independent. Within clade 1, two subdivided clades were generated. One subclade included *P. sciaenae* and *P. lateolabracis*, whereas the other subclade included *P. nemipteri*, *P. sawara* and *P. madai.* In clade 2, *P. clavaeceps* was unexpectedly included together with the four *Philometroides* species.

DISCUSSION

Morphological and molecular analyses were used to describe and compare male and female specimens of *P. sawara*, *P. nemipteri* and *P. sciaenae* with those of *P. lateolabracis* reported by Quiazon et al. (2008). Results revealed that all four are independent species. Given that males from some philometrid species have not been discovered, the addition of the reported male characteristics in this present study is valuable for species identification.

Wu et al. (2005) conducted a preliminary study on the phylogeny of nine philometrid species in China. They found a great deal of divergence in the ITS rDNA compared to 18S rDNA region. Based on this, they concluded that 18S rDNA region was more suitable for phylogenetic studies. In contrast, preliminary examination of the 18S rDNA region in the present study (data not shown) suggests that 18S rDNA region was highly conserved in the genus *Philometra* and cannot be used to clearly distinguish the species. Molecular comparisons with other ITS2 sequences in the genus *Philometra* and *Philometroides* indicated that the three *Philometra* species examined in the present study were different, particularly from *P. lateolabracis* and *P. madai* (Quiazon et al. 2008) (Table 1). The low degree of similarity (73.4%) between *P. sciaenae* and *P. lateolabracis* confirms that *P. sciaenae* is a valid species, as proposed by Moravec et al. (1998). The low degree of similarity (63.7%) between *P. nemipteri* and *P. lateolabracis* also confirms that *P. nemipteri* is a valid species and thus urgently suggests its removal from the current status of *species inquirenda*.

Philometra clavaeceps was included in clade 2 of the phylogenetic tree. This clade consisted of *Philometroides* species, suggesting that *P. clavaeceps* is more closely related to the genus *Philometroides* than the genus *Philometra.* The high level of genetic divergence between *P. clavaeceps* and other *Philometra* species may be associated with host evolution; whereas the former is parasitizing a freshwater fish, *Chanodichthys erythropterus* Basilewsky, the latter were collected from marine fishes. In this regard, it is suggested that the taxonomic position of *P. clavaeceps* be reconsidered to clarify its actual position within the family Philometridae.

The recent trend in the use of molecular tools in taxonomic studies of philometrids is important for clarification of the taxonomic position of various philometrids. This is particularly the case for those species that are repeatedly reported, such as *P. lateolabracis*. Currently, molecular tools have been used in taxonomic studies of philometrids by only few groups (Wu et al. 2005, Wijová et al. 2006, Quiazon et al. 2008). The sequencing of other reported philometrids would be very useful for the re-evaluation of the taxonomy of dracunculoids, as suggested by Moravec (2004). These

molecular data may support existing morphological taxonomy in this poorly described group. In some species where a contradiction exists between morphological and molecular data, they may draw attention to a necessity for an urgent taxonomical re-evaluation. At this stage, much work has to be done searching for and designing species-specific molecular markers for rapid and precise identification of philometrid species.

Acknowledgements. We wish to thank the Ministry of Education, Culture, Sports, Science and Technology, Government of Japan (MEXT) for financial support of this research. We thank Professor Atsuko Yamaguchi of Nagasaki University and Professor Atsushi Yamamoto of Kagoshima University for their help in collecting fish and parasite samples. We also thank Mudjekeewis D. Santos of the Laboratory of Genome Science, Tokyo University of Marine Science and Technology for the molecular analysis and improving the contents of this paper.

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Received 23 June 2008 Accepted 12 August 2008